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TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY

TRACE ELEMENT ANALYSIS IN BIOLOGICAL SPECIMENS

edited by
R.F.M. Herber and M. Stoeppler

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Edited by

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CONTENTS

Part 2 Elements

VIII

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Introduction

Detailed studies performed during the last decades on the fate and levels of trace elements in various human organs, tissues and fluids of exposed and control subjects have become a major task in toxicological, clinical, epidemiological and environmental research. This constituted a major challenge for the development of new analytical methods in this field, as well as for the improvement of existing methods.

In particular, the introduction of powerful modes of atomic absorption spectrometry such as the graphite furnace, hydride and cold vapour techniques, and recent improvements in electrochemical analytical methods by voltammetric stripping analysis and potentiometry permitted the – frequently direct – routine determination of sub mg/kg concentrations in body fluids and solid samples. From the beginning, however, large fluctuations were observed in the concentrations of many elements. After the performance of a number of intercomparison studies it became clear that there were often very large differences between laboratories in the determination of most elements. Thus, after the remarkable progress leading to advanced methods another step was needed to improve the reliability of the applied procedures at these low concentrations for e.g. toxicokinetic and toxicodynamic investigations. Two examples are the investigation of the lead content to blood serum at mg/kg or even lower concentrations and the documentation of fluctuations as a function of exposure during a working week for chromium in urine on a μg/L level.

This book intends to demonstrate the efforts of many trace analytical chemists faced with this situation in their practical daily work. Thus, the main aim is no longer the description of a variety of analytical techniques that are able to achieve the lowest possible detection limits via quite sophisticated and complicated procedures. Instead, efforts to improve reliability by well designed standard operational procedures and properly performed quality control are the core of the book.

The book offers the reader in its first part a general and as detailed as necessary introduction into the basic principles and methods, starting with sampling, sample storage and sample treatment. These steps are of utmost importance for each analytical procedure. This is followed by the description of the potential of a number of modern trace analytical methods, i.e. atomic absorption and emission spectrometry, voltammetry, neutron activation and isotope dilution mass spectrometry. The latter method is an important reference method within a general concept for quality control and the generation of reference materials which are an absolute must in this context.

Since we are aware now that the species of an element is often, on the basis of its essentiality or toxicity, much more important than the total mass of the element this aspect is also extensively treated. Quality control and the means to achieve reliable data are discussed in chapters that deal with inter- and intralaboratory surveys, and reference methods. The reference materials presently available for the treated elements and the philosophy of their production and certification are reviewed in a separate chapter. Last

but not least, the proper use of statistics as a sound basis for error recognition and data evaluation is covered in the final chapter of Part I.

The chapters in Part II are also written by scientists with a broad practical experience. They contain detailed information for the analysis in biological specimens of the thirteen trace elements that are most important for toxicological, epidemiological and environmental studies: Aluminium, arsenic, cadmium, chromium, copper, lead, manganese, mercury, nickel, selenium, thallium, vanadium, and zinc.

The editors sincerely hope that this book will, with its introduction into the basic principles and limitations of the presently available trace analytical methodology and its detailed description of reliable procedures and quality control measures, serve as a valuable aid for all those who are involved in trace element analysis. It should be especially beneficial for analysts and researchers in clinical chemistry, toxicology, biochemical and environmental research first as a general overview and second to serve as a collection of elaborated methods for the reliable determination of the above-mentioned elements and some of their species in selected (human) biological specimens.

Robert F.M. Herber and Markus Stoeppler

Chapter 1

Sampling and sample storage

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INTRODUCTION

Human specimens are analyzed for trace elements in order to find out whether there is a deficiency of an element or if there has been excessive exposure to an element. From the analytical point of view, these two aims may make a big difference as the concentrations analyzed may differ by orders of magnitude, and the analytical methods applied may thus be markedly different. Even the stability of a specimen on storage may be different, depending on the concentration of the element. In dealing with contamination — the most common source of error in trace element analysis — in the laboratory, the level of the analyte in the specimens is also a decisive factor. However, when the concentrations in the specimens are high, they tend to be so in the environment of the sample collection as well: e.g. concentrations of toxic elements are very high in the work place. Thus the risk of contamination during sampling is important irrespective of analyte level.

When trace elements are analyzed in living subjects, the specimens that are available are limited. Usually only blood, urine, faeces or hair are available. Thus the tissues or organs of most interest, e.g. target organs for a toxic effect, may not be directly sampled and analyzed. The physiological factors that affect the relationship between the concentrations of a trace element in the target tissues and in the body fluids are thus very important.

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Especially in toxicological analysis, the concentrations of elements in body fluids do not remain stable, but vary with time; therefore, one has to consider the representativeness in time. When doing post mortem analyses, the specimens are usually not limited but the distribution of a chemical within an organ may be uneven.

In the following, sources of error that are important in the process of specimen collection and storage, as well as the physiological factors that cause error in the interpretation of the analysis of trace elements in human tissues are treated.

Various aspects on sources of error in sample collection and storage for trace element analysis have been reviewed (Aitio, 1981; Aitio and Järvisalo, 1984; Anand et al., 1975, Angerer et al., 1983; Behne, 1981; Gills et al., 1974; Hops, 1977; Iyengar and Sansoni, 1988; Kosta, 1982; Kumpulainen, 1984; Sansoni and Iyengar, 1980; Stoeppler, 1980, 1983; Sunderman, 1980; Valkovic, 1977; Versieck et al. 1982).

PHYSIOLOGICAL SOURCES OF VARIATION

Blood specimens

Physiological factors that contribute to the intra-individual variation of blood constituents have been reviewed recently (Statland and Winkel, 1977, 1981; Young, 1979; Solberg and PetitClerc, 1988). Data on the effects of physiological changes on the concentrations of trace elements in blood are scanty, but it seems inevitable that physiological factors should also have similar effects on determination of their concentrations.

Distribution of water depends on posture: standing up leads to a loss of plasma water, and to an apparent increase of approximately 10% in the concentration of nondiffusible blood constituents such as proteins or cells. Posture-dependent changes may be much more marked in disease states (e.g. Eisenberg and Wolff, 1965).

Similar changes in water distribution take place locally, for example when a tourniquet is used for blood specimen collection (Statland and Winkel, 1981; Young, 1979; Solberg and PetitClerc, 1988). Most trace elements are transported in the blood bound to proteins or cells. Changes in posture and application of a tourniquet will therefore change their apparent concentrations.

Exercise — even of short duration — causes changes in blood serum constituents, possibly due to leakage of intracellular components, e.g. enzymes from muscles. Continuous training may cause hemodilution; this may in turn lead to apparently too low values of blood components. Changes caused by physical strain may even be seen in urinary excretion of trace elements, as exemplified by a 5-fold increase of the urinary chromium excretion after running for 2 h (Anderson et al., 1982a).

During pregnancy the plasma volume increases by a third; this elicits changes in the concentrations of many blood components (Hytten and Leicht, 1971; Young, 1979); lactating may also affect serum composition, as exemplified by chromium (Anderson et al., 1993).

Errors caused by physiological variation should be decreased by standardization of

specimen collection (Alström et al., 1975; Solberg and PetitClerc, 1988).

Many pathological conditions may affect concentrations of chemicals in blood or serum. Several trace elements (e.g. lead, arsenic, zinc and copper) are excreted primarily through the liver. Liver disease or cholestasis thus probably decrease their clearance (e.g. Klaassen, 1976). On the other hand, kidney damage causes retention of those elements (e.g. Al, F), whose main excretory route is through the kidneys (Marsden et al., 1979; Schiffl and Binsavanger, 1980; Wills and Savory, 1983).

Urine specimens

Usually, only 'spot' urine specimens are available for trace element analysis. Because the concentration of many analytes is dependent on the rate of urine excretion, which varies to a great extent even in healthy people (Shephard et al., 1981; Young, 1979), some standardisation for urinary excretion rate has long been used in the assessment of exposure to toxic elements (Levine and Fahy, 1945; Molyneux, 1966; Elkins et al., 1974). The most widely used approaches have been based on relative density, the concentration of creatinine in urine, and the length of the urine collection period, i.e. excretion rate. Araki and co-workers have extensively studied the correction of urine concentrations to a standard urinary flow rate of 1 mL/min in circumstance, where the water intake has been changed (Araki, 1980; Araki and Aono, 1989; Araki et al., 1990). Although this approach cannot be applied in routine trace element analysis and is not necessarily representative of other situations where the renal treatment of trace elements and water varies, it provides a useful method to compare the behaviour of the excretion of different chemicals in the urine by the following equation:

$$\log C_o = a - b * \log F \tag{1}$$

where C_o = observed concentration, F = urine flow rate mL/min, and b = chemical constant.

The correction equation will be:

$$C_s = C_o * F^b \tag{2}$$

where C_s = standardised concentration.

It can be seen from equation (2) that when the constant b approaches 0, no correction is required, i.e., the observed concentration is independent of urinary flow rate. This, however, does not seem to be true for any chemical studied (including, especially, creatinine) (Araki et al., 1990). When b approaches 1, the corrected concentration is proportional to urine flow rate, and correction to relative density is rather accurate. This is the case for mercury (Araki et al., 1990), and nickel in some circumstances (Nieboer et al., 1992). On the other hand, when b approaches 0.67, the b constant for creatinine (Araki et al., 1990), correction to creatinine excretion would seem most appropriate in routine biological monitoring. Manganese and cadmium are candidates for this approach. Chro-

mium and copper are intermediate, and thus correction to either creatinine or relative density may be applied. However, for several chemicals, interindividual variation of the constant b is very large (for example, cadmium, manganese), and consequently, no general correction method will give accurate results.

When the urine is very dilute (relative density < 1.010 or urinary creatinine <0.3 g/L) it is unlikely that any correction will give accurate results. It follows then, that the uncorrected data must be mentioned for reasons of comparison and retrieval regardless of the correction method used.

Concentrations of many body constituents show a diurnal variation. From the point of view of the standardisation of trace element analysis, it is important to note that the excretion rate of water, and of creatinine, are among such chemicals (Araki et al., 1983). Diurnal variation has also been demonstrated for chemicals such as manganese, mercury and lead (Piotrowski et al., 1975; Aitio et al., 1983; Araki et al., 1983; Järvisalo et al., 1992). In kidney damage the excretion of both water and solutes into the urine changes. The urinary excretion of cadmium is increased in cadmium-induced kidney damage. Kidney damage usually decreases the clearances of compounds that are mainly excreted through the tubuli (Foulkes, 1981; Pritchard, 1981).

Hair

Concentrations of trace elements area different in hair collected from various anatomical locations, and vary along the length of the hair (DeAntonio et al., 1982; Gibson, 1980; Grandjean, 1983; Hopps, 1977; Valkovic, 1977). History of heavy metal exposure has been elegantly elucidated from analyses of scalp hair (Grandjean, 1983); in other locations, though, this is probably more difficult because the proportion of actively growing hair at any given point in time is smaller. Variation in the rate of hair growth within an individual and between different individuals further complicates elucidation of the exposure history (Gibson, 1980; Valkovic, 1977).

Trace elements in the hair originate from the matrix proper, from sebum, sweat, and from extrinsic sources (dust, shampoos, coloring) (Gibson, 1980). The differentiation of the source has proved very difficult. In an elegant study using radiolabelled metals, Nishiyama and Nordberg (1972) were not able to differentiate between extrinsic and intrinsic cadmium, lead, mercury or methyl mercury with any washing procedure. Similar findings have been reported for arsenic (Young and Rice, 1944). Different washing procedures resulted in different, procedure-specific plateau concentrations of chromium, manganese, iron, copper, zinc, and cadmium (Assarian and Oberleas, 1977; Kumpulainen et al., 1982; Salmela et al., 1981). There was no way of knowing which of these plateaus represented the true intrinsic metal concentration of the hair. Hair cosmetics in everyday use altered the calcium, magnesium, copper and zinc content of hair; different washing procedures could not eliminate this source of error (Hilderbrand and White, 1974).

Because of these problems, hair trace element analysis is best suited for follow-up studies on individual patients (Anttila et al., 1984; Grandjean, (1983).

KINETIC SOURCES OF VARIATION

Especially toxic concentrations of trace elements are not constant, but change with time, and often show an exposure-related fluctuation. For example, the half-time of nickel and chromium in exposure to water soluble compounds in the urine is 1-2 days (Tossa-vainen et al., 1980). Thus, to be able to interpret the concentrations, the time since exposure must be standardized.

VARIATION ASSOCIATED WITH SPECIMEN COLLECTION AND STORAGE

Precipitation and adsorption

Urine, when voided, is often an oversaturated solution, e.g. urates and phosphates. In addition, cells and cell conglomerates may act as centres for crystallisation. Urine there-fore tends to precipitate on storage. Cooling or warming may cause additional precipitate formation. Bacterial infection in the urinary tract may increase the tendency to precipita-tion. Trace elements in the urine may coprecipitate with other elements, or adsorb onto the surface of the precipitates.

After [63]nickel was added to urine samples, it adsorbed on the precipitate that formed. This loss of nickel was less than 1% at pH 1, but 6% at pH 6 (Stoeppler, 1980). When urine was stored at pH 2, no loss of cadmium on to the forming precipitate was seen (Stoeppler and Brandt, 1980).

Centrifugation of urine decreased the mercury content of the liquid phase (Lindstrom, 1959). Full recovery of mercury added in urine could only be achieved if the specimen was vigorously shaken before analysis. None of the preservatives tested could prevent this loss (Trujillo et al., 1974).

Arsenic, copper, antimony, chromium, mercury, selenium and zinc were concentrated in the precipitate upon storage of acidified urine for 2 days, whereas manganese, cobalt, caesium and rubidium remained in the supernatant fraction (Cornelis et al., 1975).

Marked differences thus exist between losses of different metals on precipitates in urine. This loss must always be accounted for when sampling urine specimens. Precipita-tion is a factor to be remembered also when preparing quality control specimens in a urine matrix: the homogeneity of the quality control specimens has to be investigated carefully.

Adsorption on vial surfaces

Adsorption of metal cations onto surfaces of different types of glass or plastics from distilled, fresh, and even sea water is a well recognized problem. Acidification of the specimen has in general been used as means of prevention of adsorption (Christman and Ingle, 1976; Coyne and Collins, 1972; Robertson, 1968; Rosain and Wai, 1973; Shendrikar

et al., 1976; Shendrikar and West, 1974, 1975; Smith, 1973a,b; Struempler, 1968; Trujillo et al., 1974; Tscöhpel et al., 1980; Unger and Green, 1977).

Much less data are available on the adsorption of elements onto surfaces from blood or urine samples. Stoeppler (1980) did not detect any loss of added ^{63}Ni from urine samples onto polyethene container walls. Concentrations of nickel or chromium in urine samples, spiked with small concentrations of the metals and stored for 6 months at 4°C did not show a decrease (Kiilunen et al., 1987). The IUPAC reference method for nickel in urine calls for acidification of urine quality control samples with nitric acid and storage in polypropene tubes with a screw-cap at –20°C (Brown et al., 1981). No adsorption of cadmium onto container walls (type not specified) was seen from urine acidified to pH < 2 (Stoeppler and Brandt, 1980).

Polypropene tubes were found to be suitable for storage of serum for aluminium analysis (Bertholf et al., 1983; Leung and Henderson, 1982), whereas Vacutainer® (glass) tubes gave rise to either increases (leaching) or losses (adsorption) in the aluminium content (Bertholf et al., 1983). No change was seen in the concentration of cadmium or manganese in blood during 5 days in Vacutainer® tubes (Pearson et al., 1983). Conflicting results have been reported on the stability of blood samples for lead analysis: Although several studies indicate that lead can be stored in different plastic (polypropene, polyethene, polystyrene, polycarbonate) or glass (Pyrex, soda glass) vials especially in frozen state (Boone et al., 1979; Moore and Meredith, 1977; Subramanian et al., 1983; Wang and Peter, 1985) without losses, some studies have also reported marked losses (De Haas and De Wolff, 1981; Nackowski et al., 1979; Mranger et al., 1981).

An elevated concentration (50 μg/L) of chromium in serum remained unchanged for less than 4 days at room temperature, less than 3 weeks in a refrigerator, but more than 18 months at –10°C in polyethene or polycarbonate tubes (Anand and Ducharme, 1976).

A solution of cobalt 0.3 μg/L in appr. 0.1 molar salt solution, at pH 7, was stable for 28 days in polystyrene tubes (Barfoot and Pritchard, 1980). Blood specimens for cobalt analysis could be stored in plastic tubes for one week in a refrigerator; in longer storages deep freezing was reported to be necessary (Angerer and Heinrich, 1984).

Although the adsorption of trace elements on container surfaces from biological specimens has not been thoroughly studied, it seems that this process is not equally important as it is in the storage of water specimens.

Chemical deterioration and analyte evaporation

Little attention is usually paid to chemical deterioration and analyte evaporation when discussing the analyses of trace elements in biological fluids. However, analyses of mercury demonstrate that they should not be completely forgotten. Mercury solutions, in water, and in urine, are notoriously unstable (cf. Baier et al., 1975; Bothner and Robertson, 1975; Chau and Saitoh, 1970; Christman and Ingle, 1976; Coyne and Collins, 1972; Feldman, 1974; Gills et al., 1974; Greenwood and Clarkson, 1970; Hawley and Ingle, 1975; Lindstrom, 1959; Litman et al., 1975; Lo and Wai, 1975; Magos et al., 1964; May et al., 1980; Oda and Ingle, 1981; Taylor and Marks, 1973; Toribara et al., 1970; Trujillo et al.,

1974). The mechanisms of such losses seem to be manifold: evaporation, adsorption on container walls, and precipitation. Even diffusion through polyethene has been suggested (May et al., 1980). It seems evident that a prerequisite of loss by adsorption and evaporation is the reduction of mercury (II) to mercury (I), and subsequent disproportionation to metallic mercury Hg^0 and Hg (II) (Toribara et al., 1970). Metallic mercury is then easily evaporated, and may also be adsorbed onto container surfaces. Reduction of bivalent to monovalent mercury proceeds in many biological systems (Magos et al., 1964; Toribara et al., 1970). Acidification with, for example, nitric acid has been the classical method of stabilizing low level mercury solutions (Gills et al., 1974). With water samples preservation by means of acidification has not given unequivocally positive results (Feldman, 1974), and alternatives have been sought; for example, the addition of strong oxidizing agents such as potassium permanganate or potassium dichromate (Christman and Ingle, 1976; Feldman, 1974; Lo and Wai, 1975; Toribara et al., 1970). A combination of 5% NHO_3 and 0.01% $K_2Cr_2O_7$ was able to stabilize aqueous solutions of 1 μg/L mercury for at least 6 days. One per cent HNO_3 with 16 μg/L Au(III) had the same effect (Christman and Ingle, 1976). However, the combination of nitric acid with potassium dichromate destroys methylmercury. Therefore, if speciation of mercury is intended, the samples must not be preserved with this mixture (Oda and Ingle, 1981). Potassium persulfate was capable of preserving urinary samples for mercury analysis when the concentration of mercury was 1 mg/L (Trujillo et al., 1974). As the addition of a solid would be very convenient in routine practice, it would be worthwhile verifying this finding at low mercury concentrations.

CONTAMINATION

Contamination is by far the most important source of error in the analysis of trace elements. Trace elements are ubiquitous in the earth's crust, and tend to enter the samples in all phases of the performance of the measurement. The increasing sensitivity of analytical methods has resulted in the use of decreasing volumes of the specimens, and therefore, to decreasing amounts of the analyte in question. Thus the effect of even a μg-level of contamination is catastrophic. Recognition of contamination has also dramatically changed our views on the true concentrations of many trace elements in biological samples (see Versieck and Cornelis, 1980). Contamination may be derived from the air, from the skin of the subject or the collector of the sample; from specimen containers; from additives (anticoagulants, preservatives), and reagents used in the analysis, as well as from parts of the analytical instrumentation, such as pipette tips or neutron activation vials.

Workplace air and skin

Contamination from workplace air causes the most drastic errors in determination of toxic elements in body fluids. The reason is the high concentrations of the chemicals in workplace air, which tend to be orders of magnitude higher than, e.g., in the air in the

testing laboratories.

In addition to air-borne dust, contamination on the skin may result from sweating: Hohnadel and coworkers (1973) detected high concentrations of nickel, copper, zinc and lead in sweat collected during a sauna bath. The significance of the quantities found is not easily interpreted. However, as water from the sweat evaporates, the amounts remaining on the skin may be rather high. Another point to remember is that it is customary to explore the vein in the forearm by lightly pressing the skin with a finger. Therefore not only the skin of the subject but also the hands of the person collecting the specimens may be a source of contamination.

The skin has to be thoroughly cleaned before drawing blood samples for toxicological analysis. Washing with 0.1 mol/L hydrochloric acid, followed by rinsing with ethanol, removes lead contamination from the skin. Washing with only ethanol or ethanol and water was not sufficient for this purpose (Juselius et al., 1975). In a study of plasma lead, Everson and Patterson (1980) washed the skin successively with soapy water, alcohol, acetone, hydrochloric acid, and low-lead water.

The risk of contamination from workplace air is even greater when urine specimens are collected. They should not be collected in the workplace, but at a separate site, and only after making sure that no dust from the clothes of the worker may reach the specimen container.

Needles

By neutron-activating disposable stainless steel needles, and analyzing the resultant radioactivity in blood specimens drawn through these needles, Versieck and coworkers (1982, 1972) showed that large amounts of chromium, nickel, cobalt and manganese were leached in the first 20 mL of the blood. Damage caused by the activation process did not explain the findings. With a similar technique, Lakomaa (1980) demonstrated that specimens of cerebrospinal fluid obtained through conventional needles could not be used for assessing chromium or nickel concentrations because of contamination from the needle. Concentrations of chromium in the blood obtained through disposable stainless steel needles were 4 times higher than those in blood obtained through a plastic catheter (Kumpulainen et al., 1983); the first 20 mL of blood collected through a stainless steel needle contained 8 times more chromium than the next three 20-mL portions (Versieck et al., 1980). Siliconisation of the needle seemed to abolish chromium contamination (Veillon et al., 1984; Ericsson et al., 1986). Siliconisation of the needle may decrease also the nickel contamination: Serum nickel values in blood samples obtained through disposable needles were two times higher than those from samples obtained through plastic ca-theters (Sunderman et al., 1984), while no such effect was seen when siliconized needles were used (Bro et al., 1988). Elevated serum manganese concentrations were also re-ported after collecting samples through stainless steel needles (Hudnik et al., 1984) while no leaching of aluminium, cadmium or cobalt was detected from needles (Gardiner et al., 1981; Frech et al., 1982; Bertholf et al., 1983; Andersen and Hogetveit, 1984; Hudnik et al., 1984). However, Parkinson and co-workers (1982) have reported that stainless steel

venipuncture needles used by them were randomly contaminated with aluminium.

Surgical instruments

Versieck and coworkers (1972, 1973) have demonstrated that Menghini needles used for transcutaneous liver biopsy introduce a marked error in the analyses of cobalt, nickel and chromium, and a less pronounced contamination (doubling or less) in the analysis of silver and tin. In operative biopsies using surgical blades, significant contamination was detected only for nickel and chromium. On the other hand, biopsy forceps did not cause significant contamination in the analysis of small (10 mg) specimens of tonsillar tissue for their nickel content (Torjussen et al., 1977). Absence of cadmium and manganese contamination from Menghini biopsy needles was recently confirmed by chemical analyses (Hudnik et al., 1984). Quartz (Gerhardsson et al., 1984), glass (Hudnik et al., 1984; Morgan and Adams, 1980), titanium (Zober et al., 1984) and plastic (D'Haese et al., 1985) instruments have been used in collecting tissue specimens for trace element analysis, in order to avoid contamination.

Contamination from anticoagulants and preservatives

Information on the contamination resulting from the use of anticoagulants is very scanty. Lead in heparin does not contribute significantly to the concentration of lead in whole blood, whereas for lead in plasma it does, as the concentration of lead in plasma is very low (Everson and Patterson, 1980; Cavalleri and Minoia, 1981). Sodium citrate and lithium heparin were reported to contain too high concentrations of aluminium (d'Haese et al., 1985), while potassium EDTA from one source – but not from another – could be used for plasma aluminium analyses (d'Haese et al., 1985; Paudyn et al., 1989). Potassium EDTA from a further source was found suitable for the analysis of cobalt in whole blood (Angerer and Heinrich, 1984); however, in an analysis of a large number of trace elements, and several anticoagulants, contamination was most frequently encountered when EDTA was used (Paudyn et al., 1989).

Although analytical data on other metals in anticoagulants are not available, it would seem that all anticoagulants are a probable source of contamination.

Hydrochloric and nitric acids have been used as preservatives for urine specimens for metal analyses, and mineral acids are extensively used in graphite furnace analysis of trace elements in biological specimens (e.g. Gills et al., 1974; Stoeppler and Brandt, 1980). Historically, concentrations of trace elements in commercially available acids have been incompatible with analysis of trace elements in biological samples (Kuehner et al., 1972). However, present commercial ultra pure hydrochloric, nitric, sulphuric and perchloric acids have been reported to be suitable for trace element analysis in urine without further purification (Golimowski et al., 1979; Brown et al., 1981; Veillon et al., 1982).

Contamination from glass and plastic ware

Glass (cf. Tschöpel and Tölg, 1982; Veillon et al., 1982) and various plastics (Karin et

al., 1975; Moody and Lindstrom, 1977; Robertson, 1968) contain varying amounts of practically all elements, and these may leach into water, acids, as well as blood and urine specimens. Even high purity quartz test tubes were a source of contamination of aluminium, chromium, and molybdenum (Ericsson et al., 1986). It seems that no commercially available container should be used for storage of specimens for trace element analysis without prior cleaning. A variety of cleaning methods has been devised (Karin et al., 1975; Kuehner et al., 1972; Laxen and Harrison (1981); Mikac-Devic et al., 1977; Moody and Lindstrom, 1977; Robertson, 1968). Laxen and Harrison (1981) have compared 13 different published cleaning methods, including different concentrations of nitric, hydrochloric and perchloric acids, and combinations of them, as well as the use of nonionic detergents, and also rinsing with distilled water only. They concluded that for analysis of zinc, cadmium, lead and copper in fresh water, soaking in 10% nitric acid for 48 h was the most appropriate method of cleaning.

Evacuated blood collection tubes, and especially their rubber stoppers, have been notorious for their contamination with metals, which was first recognized with zinc, lead and cadmium (Helman et al., 1971; Juselius et al., 1975; Nackowski et al., 1977; Nise and Vesterberg, 1978; Lecomte et al., 1979, Guillard et al., 1982; Pearson et al., 1983; Narayanan et al., 1986). At present, many manufacturers market evacuated tubes specifically destined for lead, or more general trace element analysis. In many instances they have been found suitable for trace element analysis, but there may still be differences between the different brands, and analyses at lowest levels may still be contaminated (Helman et al., 1971; Nackowski et al., 1977; Gorsky and Dietz, 1978; Nise and Vesterberg, 1978; Smeyers-Verbeke et al., 1980; Guillard et al., 1982; Leung and Henderson, 1982; Bertholf et al., 1983; Pearson et al., 1983; Narayanan et al., 1986).

It seems advisable that the laboratory doing the determination should provide the specimen containers; this helps to diminish contamination risks. Commercial evacuated tubes may introduce contamination to blood specimens. Although this risk seems smaller if dedicated trace metal brand tubes are used, absence of contamination should be verified before starting use of commercial tubes.

LOSS OF WATER

Loss of water by evaporation results in apparent increases in the concentrations of the chemical studied. Water may be lost through the container walls, or through leaky closures; therefore the results may be quite variable from one container to another. Generally, errors introduced by water loss are minor, and do not affect everyday routine analyses. However, in specimens with long storage times (especially quality control materials!) such loses may become significant. Annual losses of water from polyethene, polypropene, high density polypropene, Teflon-FEP, and glass containers were less than 0.5% (Curtis et al., 1973; Moody and Lindstrom, 1977). Losses from polyvinyl chloride were appr. 0.5%, those from polycarbonate appr. 2% and from polymethylpentane 1% annually (Moody and Lindstrom, 1977).

REDISTRIBUTION OF THE ANALYTE

The concentration of manganese is more than 20 times higher in erythrocytes than in plasma (Versieck et al., 1974; Milne et al., 1990; Järvisalo et al., 1992). The ratio for lead is 50-100 (Cavalleri et al., 1978; Everson and Patterson, 1980; DeSilva, 1981; Manton and Malloy, 1983; Araki et al., 1986; Kehoe, 1987; Ong et al., 1990); for zinc it is approximately 10 (Ishihara et al., 1984). It is thus evident that destruction of cells leads to gross elevation of the concentration of such elements in plasma/serum. Plasma can be separated more rapidly than serum from cells and the procedure is more gentle toward the cells. Therefore, plasma is to be preferred over serum when elements with an unequal distribution in the blood are analyzed, although the addition of anticoagulant adds a risk of contamination (see above). Hemolysis may give rise to analytical interferences, e.g. by liberation of considerable amounts of iron into the serum. Iron interferes with, e.g., the determination of nickel by graphite furnace atomic absorption (Brown et al., 1981).

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Chapter 2

Sample treatment of human biological materials

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INTRODUCTION

The number of elements found to play a metabolic role in the human body has been continuously increasing. They are present in a varied range of concentrations and include
   a) those present at comparatively high concentrations, e.g. Na, K, Ca, Mg
   b) those present at mg/kg and lower levels and acting as carriers of enzymes, e.g. Zn, Cu, Ni, Mn, Se
   c) those present as undesirable contaminants in a variable range of concentrations, e.g. Pb, Cd, Hg
   d) others eg. Cr, Au, Pt, Li, Al intentionally introduced into the body for treatment of a specific disease.
The quantitative analysis of human biological material has assumed great importance in view of the need to evaluate the concentrations of various elements within a range of concentrations in clinical specimens.

The trace element content of a given biological material can be invariably estimated from the intensity of an analytical signal originating from the element in a sub-sample which is representative of the total material. It is therefore obvious that this sub-sample should not only be a true representative of the total material, but should also give rise to an error free analytical signal. Sample treatment, therefore, includes all steps involved in obtaining such a sub-sample for measuring the analytical signal through the black box of an analytical instrument.

The general steps involved in the treatment of biological materials and the sources of error in each were defined by Sansoni and Iyenger (1978). Sampling and storage of biological specimens have already been dealt with in Chapter 1. In this chapter we shall be concerned with transformation of the preserved/sampled specimens to a form suitable for obtaining an error free analytical signal.

BASIC CONSIDERATIONS

It is obvious that reliable and reproducible results can only be expected if the sub-sample has a mean composition, similiar to the totality of the investigated material, as regards the analyte elements.

This assumes sample homogeneity, no elemental loss or contamination while obtaining the sub-sample and finally the ability of the sub-sample to produce an error free analytical signal in proportion to the analytes in the totality of material. The nature of the sample material, the range of concentration of the analyte in the material, the analytical method to be used for measuring the analytical signal, and lastly the method for ashing and or dissolution of the sample, therefore, form a basic and important consideration in sample treatment of human biological materials (Sansoni and Iyenger, 1978; Merchandise, 1987; Yin and Wang, 1987).

Nature of biomedical samples

The human biological materials may be solid (bone, teeth, hair, nails, tissue), semi-solid (blood, faeces, viscera) or liquids (body fluids). Treatment of the solid samples usually demands some extra steps during sample manipulation, e.g. particle size reduction, homogenisation, sub-sampling etc. Heterogeneous liquid phases, e.g. blood and certain body fluids, additionally need stabilisation and homogenisation so as to avoid occurrence of any changes in their composition, prior to actual analysis (Anand et al., 1975). It is also advisable to keep the total number of transfers to a minimum, and use accessories made of non-wettable and inert materials in case of the liquids.

Problems of contamination

A priori knowledge of the approximate composition of bio-material with respect to the elements to be analysed is an important consideration in sample treatment. This helps to maintain the working conditions optimal to the concentration levels being sought. The factors which can cause sample contamination ought to be controlled in order to minimise the contamination. Some of these factors include the laboratory environment, the apparatus, the reagents and the analyst himself. Other potential sources of contamination may comprise cross contamination from an accompanying interferent, e.g. blood while estimating iron in serum or in the soft tissue. It is also extremely important to identify and isolate external contamination of the sample material, e.g. from dust and the laboratory environment.

Method of analysis

The method to be employed for measurement of the analytical signal largely determines the form of the sub-sample and, consequently, the extent and type of sample preparation required. Nuclear activation methods, x-ray fluorescence techniques, graphite furnace atomic absorption, classical emission spectroscopy and many mass spectrome-

tric methods do not require that the sub-sample necessarily be in the form of a solution. Solid sampling analysis (using either a graphite furnace or an inductively coupled plasma) can be extremely useful for analysis of biological materials due to a number of reasons. It is possible to eliminate the time consuming destruction step thus reducing the risks of contamination as well as loss of analyte elements (Herber, 1992). Determination of various elements in human biological materials using solid sampling has been summarised by Herber (1992). The important consideration, however, is the need of obtaining the sample as an homogeneous solid, even though proper statistical treatment of data may enable accurate results to be obtained on samples showing micro-hetrogeneity (Kurfürst, 1991). The sample preparation in this case would invariably involve only homogenisation, drying, or in certain cases, dry ashing of the sample at a higher temperature to preconcentrate the analytes. The attributes for trace element solid sampling analysis have been discussed by Van Loon (1983). It should be noted that the inorganic matrix composition of the samples is more important than the organic matrix when solid sampling analysis is employed (Herber, 1991). Solid sampling of biological materials in AAS is by now being used as a routine procedure (Lücker et al., 1992; Nordahl et al., 1990; Schauenberg et al., 1992)

A variety of other techniques eg. voltammetry, flame atomic absorption spectrometry, flame emission spectrometry, ICP-atomic emission, ICP-fluorescence and ICP-mass spectrometry are almost invariably applicable to aqueous solutions, though solid sampling using ETA technique with the ICP-MS, is well established by now (Baumann, 1992; Voellkopf et al., 1992). If the analytical technique employed is amenable only to a sub sample in solution, or is sensitive to the sample matrix, additional steps for dissolution and removal (partial or complete) of the biological matrix become unavoidable. We shall be mainly concerned in this chapter with these steps.

Ashing in sample preparation for bio-materials

The elements in biological systems are usually present in a bound form, along with a million to billion-fold excess of the organic compounds (Sansoni and Panday, 1981). The elimination of organic matter and mineralisation of these elements is therefore not only desirable, but very often forms a crucial step preceding the subsequent steps of separation and analytical measurement. This is due to the fact that organic constituents usually interfere to a varying degree during these steps, according to the technique being employed. The trace elements are either eluted from the matrix into an aqueous solution or separated by volatilisation of the organic matrix. This latter step, usually termed ashing, comprises the removal of organic matter by conversion of the latter into a suitable gaseous component. The inorganic residue left behind consists entirely of the analytes of interest. An ideal ashing method to be employed in trace analysis should be capable of (a) avoiding all contamination (b) retaining all the analyte elements quantitatively in the inorganic residue and (c) completely removing all organic components of the sample. It must be emphasised however, that ashing and dissolution are not synonymous although they might very often achieve the same objective.

General methods for ashing and dissolution

General methods for dissolution or ashing of biological materials for subsequent trace element analysis could be classified into dry and wet ashing techniques (Bock, 1979). A further sub-division can be made according to the temperature and/or pressure employed during the decomposition step. Dry ashing can be carried out in the stationary or streaming phase with the help of a variety of oxidants. Wet decomposition is usually achieved using strong mineral acids or oxidants. The nature of the oxidant invariably determines the extent of ashing. Common approaches employed for sample preparation of biological materials are listed in Table 1.

SAMPLE TREATMENT OF HUMAN BIOLOGICAL MATERIALS

Human biological materials to be investigated include (a) hard calcified tissues, e.g. bone, teeth, other calcified formations; (b) semi-hard tissue, e.g. hair, nails; (c) soft body tissues; and (d) various biological fluids and secretions in the human body. The treatment of each of these materials varies from one material to another and, as stated earlier, is often determined by the instrumental method to be employed for measuring the analytical signal, the elements to be determined and the concentration levels at which these are present. For the purposes of this discussion, it shall be generally assumed that the analytical techniques employed include atomic absorption spectrometry both with (F-AAS) as well as with a furnace (GF-AAS), neutron activation analysis (NAA), flame emission spectrometry (FES) voltammetric methods and the three inductively coupled plasma spectrometric methods viz. ICP-atomic emission spectrometry, ICP-mass spectrometry and ICP-atomic fluorescence spectrometry. The sample preparation of biological methods for all ICP techniques is usually similar (Guo, 1989).

Calcified tissues

Teeth

Teeth samples present difficulties in obtaining a uniformly homogeneous sample because of differences in the composition of enamel and dentin. It is therefore often necessary to separate these components. The extracted sample is washed with suitable detergents and the adherents are removed manually (Chatman and Wilson, 1975). The sample is cleaned further by polishing with either a slurry of silicon carbide in a rubber cup (Curzon and Losee, 1977) or other polishing agents, e.g. pumice stone, alumina (Retief et al., 1974; Losee et al., 1974; Malik and Fremlin, 1974). It is finally washed thoroughly with distilled water and dried for several hours in an oven at an elevated temperature (Helsby, 1974; Burkitt et al., 1975).

The separation of dentin and enamel is usually carried out mechanically by chipping the enamel in a mortar made of an inert material (Helsby, 1974; Retief et al, 1974) or by grinding with a diamond disc (Steinnes et al, 1974; Stack et al, 1977). A carbide steel

TABLE 1

CLASSIFICATION OF ASHING METHODS FOR HUMAN BIOLOGICAL MATERIAL IN TRACE ELEMENT ANALYSIS

1. DRY ASHING	2. WET ASHING	3. Others
1.1 High Temperatures	**2.1 High temperatures**	**3.1 High temperatures**
1.1.1 Combustion with air/oxygen	2.1.1 Normal pressure	3.1.1 Oxidative fusion
1.1.1.1 Stationary system: A. Open muffle furnace under atmospheric pressure B. Oxygen flask C. Oxygen bomb, high pressure	2.1.1.1 Oxidising mineral acids: A. HNO_3 B. H_2SO_4 C. $HClO_4$ D. HNO_3/H_2SO_4 E. $HNO_3/HClO_4$ F. $HNO_3/HClO_4/H_2SO_4$	3.1.2 Oxidation in acid vapour 3.1.3 Oxidation with ozone **3.2 Low Temperatures**
1.1.1.2 Streaming system: A. Oxygen/air stream in combustion tube B. H_2/O_2 - flame in a closed cooled system C. Cool finger (Trace-O-mat)	2.1.1.2 Hydrogen peroxide: A. 30% H_2O_2 B. 50% H_2O_2/H_2SO_4	3.2.1 Enzymatic 3.2.2 Radiative decomposition
1.1.2 Pyrolytic decomposition: A. Heating under inert gas eg. Ar, N_2 in graphite furnace B. Reduction with H_2 at high temperature C. Carbonisation	2.1.2 High pressure: A. Oxidising mineral acids in teflon bomb B. Hydrogen peroxide in bomb C. Knapp High Pressure Asher (HPA) D. Microwave oven digestion	3.2.3 Solubilisation (mineral acids, tissue solubilisers)
1.2 Low Temperatures	**2.2 Low Temperatures**	
1.2.1 Plasma of oxygen gas: A. Radiofrequency electrical fields B. Microwave electrical fields	2.2.1 OH radicals from H_2O_2/Fe^{2+} at $100 - 220°C$ (Fenton's reagent): A. Open beaker B. Closed system	
	2.2.2 Mineral acid extraction	

round bur (Losee et al, 1974) has also been used and contamination from a number of metals has been stated to be absent. However, it would be preferable to use a more inert material. The enamel is separated from dentin using a flotation method which consists of mixing the ground sample of dentin and enamel with a heavy liquid, ultra-sonification of the mixture and its subsequent centrifugation (Manly and Hodge, 1939; Lakomaa and Rytömaa, 1977).

Very small samples of dentin can also be etched chemically using mineral acid attack (Dolinsek et al., 1975; Munksgaard and Bruun, 1973) or by polishing with inert surfaces, e.g. alumina (Brudevold et al., 1968; Wei, 1973).

The separated enamel and dentin usually need to be homogenised. This can be carried out by grinding in an inert agate mortar directly or after fracturing under liquid nitrogen temperature (Needleman et al., 1972; Iyenger and Kasperek, 1977) to obtain a uniformly homogeneous sample for analysis by NAA (Steinnes et al., 1974; Retief et al., 1974), SSMS, spectrographic techniques (Losee et al., 1974) as also by the GF-AAS technique (Langhmyr et al., 1975; Lücker, 1992).

If, however, the sub-sample is desired in the form of a solution, dissolution or ashing of the sample must be carried out. Small samples of dental enamel can be dissolved in a single acid (Dolinsek et al., 1975; Burkitt et al., 1975; Lakomaa and Rytömaa, 1977; Shapiro et al., 1973). A mixture of the mineral acids is however needed in case of the whole tooth or dentin which contain higher fat and organic matter. Acidification of dentin precipitates protein which needs to be decomposed with a strong oxidising agent. It is preferable, therefore, to dissolve dentin with a mixture of nitric and perchloric acid (Locke-retz, 1975). An alternative approach, consisting of initial dissolution followed by dry ashing in a muffle furnace, has also been reported (Chatman and Wilson, 1975). A microwave oven based digestion approach has been described for dental specimens using a ternary mixture of nitric, perchloric and sulphuric acid (Barrett et al., 1978). However, the use of sulphuric acid should not be advocated in the case of calcified tissues.

Dry ashing of dental samples can be carried out in a muffle furnace at temperatures not exceeding 500°C when the elements having moderate volatility (e.g. Zn, Pb) are quantitatively retained. However, it cannot be recommended when volatile elements (Cd, Se, Hg) are to be determined. The use of low temperature ashing in an oxygen plasma (LTA) has not been reported for dental samples but the preliminary studies carried out in the author's laboratory showed that the technique is slow and is only effective if combined with initial wet dissolution using nitric acid.

Bone

The bone samples are usually kept frozen at temperatures below -10°C. The big samples can be cut into smaller pieces using a Jeweller's saw, or can be broken into small pieces by cooling in liquid nitrogen and subsequent fracturing. The bone marrow is removed using a hot water jet wash (Blotcky et al., 1978; Hislop et al., 1973). The samples can be defatted with acetone and ether using at least two changes of the solvents over a 48 h period (Leriem et al., 1975). The adhering flesh and periosteum should be removed

using a scalpel of inert material and the sample dried in air at 50–60°C to a constant weight. The pulverisation should be carried out in an inert grinding mill such as the teflon ball mill described by Sansoni and Iyenger (1978).

The dissolution of bone has been carried out using both dry as well as wet ashing procedures. Estimation of magnesium (and possibly many more elements) could be carried out in bone samples after dry ashing these specimens at 800°C (Hunt, 1969). However, considerable discrepancy exists on the losses of various trace elements in bone on dry ashing (Hislop and Parker, 1971). Many elements of moderate volatility, e.g. Pb, are retained almost completely when ashing is carried out at temperatures not exceeding 550°C (Hislop and Parker, 1971; Panday et al., 1981; Petrow and Cover, 1965) in platinum crucibles. The question of ashing temperature and the crucible material is open to some doubt, since lead losses were higher in porcelain crucibles (Garlicka and Doniec, 1984) compared to those in the platinum ones at a temperature of 800°C. It is therefore, advisable to carry out the dry ashing of bone at lower temperatures, followed by a wet digestion with mineral acids. A mixture of nitric and perchloric acid for wet digestion of trabecular bone, after the same had been dry ashed at 450°C, was found useful (Panday et al., 1981) for determining a number of elements by F-AAS. The dissolution of residual ash with a (3:1) mixture of nitric acid and hydrogen peroxide has been found effective in estimating a wide range of elements by ICP-mass spectrometry (Sansoni and Panday, 1991).

The ashing of calcified tissues employing the LTA technique has the principal advantage that losses of the volatile elements occur to a much lesser extent than is the case with conventional ashing techniques. It is also probable that the micro-structure remains unchanged during ashing by LTA. However, the method is slow and amenable to small sample amounts only.

Wet ashing procedures using mineral acids or other oxidising agents would be expected to yield higher recoveries of elements, due mainly to lower temperatures and mineralisation of the volatile species. Some investigators (Blotcky et al., 1978; Garmestani et al., 1978) were able to dissolve bone samples in concentrated nitric acid prior to analysis for certain elements. However, a mixture of nitric and perchloric acid (Hislop et al., 1973) or another oxidising agent should be recommended (Mahanti and Barnes, 1983).

The low temperature wet ashing by Fenton's reagent would be particularly effective in digesting bone samples because of the smaller amounts of fat (Sansoni et al., 1971a,b).

Urinary and other calculi

The determination of trace elements in urinary calculi is gaining importance in view of studies on the mechanisms of their formation. These human biological specimens usually have a low organic content but are rich in phosphate, calcium and magnesium and also have varying amounts of silicates. The specimens have been studied directly by non destructive techniques, e.g. instrumental neutron activation analysis (Jacimovic et al.,

1977). A novel method using charged particle activation required only smoothing of the sample surface (McConville, 1974).

In view of their non-homogeneous nature, the specimens are crushed and obtained into a powder form using a vibrating mill or in an agate mortar (Strübel et al., 1987). The powdered samples can be directly introduced onto a L'vov platform for measurement of a number of heavy metals by GF-AAS. If the samples are to be obtained in solution, the ground specimen is moistened with a mineral acid, e.g. 6N HCl and heated to dryness. The organic components are decomposed by adding a few drops of perchloric acid followed by heating to white fumes (Spooner and Crassweller, 1972). The final contents can be dissolved in a dilute mineral acid for aspiration into a flame or an ICP.

It is best to fuse the sample with a fusion mixture (Burr, 1976) when silicate silica is to be determined. The mixture of the sample and the fusion mixture were heated to redness for 10 min, cooled, transferred to a volumetric flask and made upto volume with citric acid. The procedure can not be suggested when other trace elements are to be determined in the matrix.

Decomposition of these stone formations using nitric acid and perchloric acid, followed by a few drops of hydrofluoric acid in a microwave oven should be the method of choice for trace analysis of these stone formations using most analytical methods requiring the sample in solution.

Semi hard tissue

Hair and nails form two important tissues in this group. These tissues require some pre-treatment prior to actual sample dissolution due primarily to the surface contamination of these materials from the environment.

Hair

Hair offers a good way of discerning long term variations in trace element concentrations since (a) the concentrations of most trace elements are relatively higher in hair compared to those in other human materials, (b) the specimens can be collected quickly and easily and (c) the material is inert as well as homogeneous (Laker, 1982). The removal of surface contamination from hair samples before analysis is one of the major problems encountered by an analyst if the surface contamination has to be distinguished from the trace elements incorporated in the human body (Takeuchi et al., 1979; Clark and Wilson, 1974). There are merits and demerits for the various washing procedures described in the literature for removal of the surface contamination from hair samples (Assarian and Oberleas, 1977; Bate, 1966; Hambridge et al., 1972; Harrison et al., 1969; Hildebrand and White, 1974; Salmela et al., 1981; Schroeder and Nason, 1969).

Most washing procedures fall into the following categories of a wash (IAEA, 1978) with:
   a) acetone-ether
   b) hexane-ethanol
   c) a detergent

d) EDTA

The samples of hair are invariably soaked for 10–30 min in the first solvent with occasional shaking, decanted and then soaked similarly in the next solvent. It is general practice to repeat the wash several times even though the losses of several trace elements continue to rise with each wash. The samples are finally rinsed repeatedly with distilled water and dried at 50–60°C in a clean environment for several hours. The losses of trace elements depend on the washing solvent as well as the element. Large variability has been found for copper after washing by different procedures (Assarian and Oberleas, 1977) although zinc was found to be unchanged during most washing procedures (Hildebrand and White, 1974). The use of hot boiling water or certain non-ionic detergents would be efficient in removing surface contamination as well as in minimising the loss of trace elements from hair specimens. It is difficult to conclude which procedure is best suited for all the elements, but there is no doubt that chelating procedures, e.g. the use of EDTA, are prone to higher loss of trace elements, and almost certainly remove more than the surface contamination.

Further preparation of the hair specimens may involve (a) powdering for direct solid sampling (e.g. in GF-AAS or INAA), or (b) dissolution. The powdered material should preferably be obtained by cooling under liquid nitrogen and grinding in a teflon ball mill. Dissolution of the hair samples and solubilisation has been carried out using aqueous solutions of certain bases, e.g. Soluene, Lumatom, which are available commercially. Afew mg of the sample is usually incubated at 50–70°C for a few hours and the contents diluted with a solvent, e.g. toluene or alcohol. Removal of organic components from the samples can be achieved only if dry or wet ashing procedures are employed. A dry ashing temperature of 400–450°C should suffice to bring the samples to a white ash. The addition of a few drops of suprapure conc nitric acid after a pre-ashing period of 4–5 h, facilitates the ashing (Friel and Nagyuen,1986). The final ash can be dissolved in 6N nitric acid and made to volume with ultra-pure water.

Low temperature ashing in an oxygen plasma (LTA), first described by Gleit and Holland (1962), is an effective and rapid method for ashing hair samples which invariably need to be analysed for volatile elements.

Wet digestion has usually been the method of choice for hair samples (Friel and Nagyuen, 1986; Lee Jones et al., 1988). The digestion of hair samples can be carried out with a single mineral acid in case the samples are to be aspirated into a graphite furnace or a flame for AAS determinations. However, complete removal of organic matter can only be achieved if a strong oxidising agent such as perchloric acid or hydrogen peroxide is employed. A mixture of mineral acids should, therefore, be preferred for digesting hair. Care should be taken so as not to char the mixture if sulphuric acid is employed for attaining an elevated temperature. It is recommended that the use of sulphuric acid be avoided in trace analysis, because of (a) the formation of insoluble alkaline earth sulphates, (b) the presence of a large number of interference effects during various analytical measurements and finally (c) the not so pure nature of this acid.

Nails

The nail samples are similar to hair in many respects but the total amount available is usually small. The samples are invariably collected using Ni-coated unalloyed steel clippers or scissors. The specimens should be scraped further using blades made of quartz or forceps coated with teflon. Cleaning of the samples to remove fat, sweat and dirt can be carried out by soaking in suitable solvents, e.g. acetone, ethyl alcohol (Mahler et al., 1970) or in a non-ionic detergent (Harrison and Tyree, 1971). The samples should then be rinsed with a copious flow of deionised water and finally dried in a clean atmosphere at a temperature of 60–90°C.

Dissolution of nails and removal of the organic matter can be achieved using procedures applicable to hair samples (Helsby, 1976). The micro-samples can often be directly introduced into the graphite furnace for analysis by GF-AAS (Harrison and Tyree, 1971; Sohler et al., 1976) or after digestion with a mineral acid. Solubilisation with tissue solubilisers, e.g. TAAH is useful for measurements by AAS (both flame and GF). The complete removal of biological matter, however, necessitates use of stronger oxidants such as perchloric acid or hydrogen peroxide. The digestion of nail samples in a microwave oven (Abu-Samra et al., 1975) is very effective if a few drops of sulphuric acid are added to the digestion mixture.

Removal of organic matter from the nail samples prior to mineralisation can also be effectively achieved using either the low temperature ashing (LTA) or the cooling finger apparatus of Raptis et al. (1982).

Soft tissues

The soft biological tissue to be dealt with includes autopsy as well as biopsy specimens from various human body organs, e.g. liver, kidney, brain, lung, placenta and other connective tissues. No unified approach for handling of these tissues has been established despite the numerous problems during preparation of these samples for analysis. However, several reports are available on typical body tissues, e.g. kidney (Livingstone, 1971), liver (Iyenger and Kasparek, 1977).

A primary difficulty is due to the interfering components from the surrounding tissues such as the connective tissues, blood vessels, nerves, hair, bone marrow, residual blood and extra-cellular fluids which are intricately mixed with the sample material. These components invariably result in wide variations for some elements, e.g. iron (Iyenger, Kollmer and Bowen, 1978). Thawing of the samples helps in partially removing the interfering components, e.g. blood. A quick deionised water wash might sometimes be helpful. This may entail the loss of body fluids which could be a source of negative bias. The problem is generally more severe in the case of biopsy samples because of the smaller sample sizes available. Another problem which might occur is the determination of exact wet weights for these samples. Freeze drying of biological tissues and a careful screening of the specimens might help reduce several problems (Sansoni and Iyenger, 1978). Homogenisation is liable to introduce contamination from the materials (van Grieken et al.,

1980). However, the brittle fracture technique in which the specimen is placed in a telfon mill, cooled in liquid nitrogen and vibrated (Iyenger and Kasparek, 1977) is efficient in minimising contamination. Sample lyophilisation is another clean technique, although some losses of elements are known to occur (Iyenger and Sansoni, 1980; Fourie and Peisach, 1977). The application of microwave heating for drying would seem to be promising and needs to be investigated with respect to losses of volatile trace elements.

Solid sampling techniques enable direct analysis of the homogenised tissues. A number of such applications for analysing solid biological tissues have been reported (Chakrabarti et al., 1980; Lundgren and Johansson, 1974, Nordahl, 1990). However the dried tissue invariably needs to be solubilised for trace element analysis using techniques requiring the sample in a solution form (Fry and Denton, 1977; Hohl et al., 1989).

The use of commercially available tissue solubilisers, e.g. Lumatom, Soluene or TMAH (Shimizu et al., 1988) is a simple solubilisation technique for bio-materials, e.g. tissue. However, it suffers from the disadvantages of limited time stability, prolonged digestion times (sometimes up to 48 h) and non-applicability to polarographic methods. In any case, it seems to be suited to measurements by AAS techniques (both flame and GF). Another effective approach suggested for tissue solubilisation is enzymatic decomposition (Kracke and Bunzl, 1978) and can be applied to the determination of a number of elements in biological tissue.

A number of dissolution techniques which effectively elute the trace elements of interest include leaching with single mineral acids or a mixture of them (Johnson, 1976; Maurer, 1977). The extracts are suitable for measuring a large number of elements including Bi, Cd, Cr, Co, Cu, Pb, Mn, Ni, Ag, Tl and Zn. The speed and effectiveness of solubilisation can be enhanced using pressurised vessels for dissolution (Adrian, 1973; Stoeppler and Backhaus, 1978; Iida et al., 1980; Scheubeck et al., 1979). Complete removal of the biological matter is desirable in trace element analysis of biological tissues. It helps guard against the possibilities of interference during pre-concentration of the analyte as well as the instrumental measurement. Most important methods for removal of biological matrix from the tissue include dry ashing in a muffle furnace (Tam and Conacher, 1977; Versieck et al., 1979; Menden et al., 1977; Tam and Lacroix, 1979; Adeloju et al., 1984), low temperature ashing in the oxygen plasma (Gleit and Holland, 1962), wet ashing with mineral acids under pressure (Knapp et al., 1981; Kotz et al., 1979) and in the microwave oven (Kingston and Jassie, 1986; Matusiewicz et al., 1989; Pinheiro et al., 1990).

The use of the conventional dry ashing technique, however, is decreasing in popularity due to possible loss of elements at elevated temperatures despite encouraging reports (Versieck et al., 1979; Blanusa and Breski, 1981). The carbonisation technique reported recently (Satake and Uehiro, 1985) for biological materials helps reduce the losses of volatile elements. This does not however cause complete destruction of organic material. A combination of charring at relatively low temperature (375°C) in a muffle furnace followed by oxidation with H_2O_2 has been used for complete recovery of I, Cu, Mn, Fe, Zn, Cr, Mg and Ca in endogenously labelled tissues and NBS reference materials (Hill et al., 1986).

Combustion in a closed stationary system (Belcher and Macdonald, 1958) has been found useful for a limited number of elements and low recoveries of many elements have been reported due to a variety of causes (Desai et al., 1984).

The modification of this technique for dry ashing small amounts of biological specimens (Raptis et al., 1982; Raptis et al., 1983; Tölg, 1977) is to be preferred for biopsy specimens. An apparatus is also available commercially (Knapp et al., 1981).

Dry ashing of organic matter under the influence of a high frequency field (Gleit and Holland, 1962) has been applied to standard reference materials (Lutz et al., 1977), bovine liver (Pallon and Malmqvist, 1981) and other biological materials (Han et al., 1982). It is however most effective when the biological tissues have been pulverised to a fine powder. Investigations in our laboratory show that ashing periods exceeding 60 hours are needed before destruction of the organic matter can be considered complete.

Wet ashing of biological tissue is usually preferred, due to lower losses of elements and comparatively smaller ashing times (Haas and Krivan, 1984). Incomplete digestion often suffices for determining elements, e.g. Ag, Cd, Bi, Co, Cr, Cu, Mn, Fe, Ni, Pb, Tl, Zn by atomic absorption and flame emission spectrometry (Maurer, 1977; Hinners, 1975; Jarnstrom et al., 1983; Johnson, 1976). For this reason HNO_3 has been found to be the most effective agent (Clegg et al., 1981). Complete digestion of biological materials would require the use of strongly oxidising mineral acids, H_2O_2, or a suitable combination of these reagents. The completeness and speed of digestion can be enhanced using pressurised vessels (Adrian, 1973; Stoeppler et al., 1979; Knapp, 1988; Würfels and Jackwerth, 1985). The use of microwave technique (Aysola et al., 1987; Barrett et al., 1978; Kingston and Jassie, 1986) is also quite effective in mineralising biological tissue. The precautions to be observed with the use of powerful oxidising agents, e.g. $HClO_4$, H_2O_2, have been summarised in many excellent reviews (Gorsuch, 1970; Tsalev and Zaprianov, 1983; Tsalev, 1984). It has therefore been often avoided in digesting biological materials (Reamer and Veillon, 1983).

The possibility of destroying biological tissue using Fenton's reagent makes this method (Kreuzer et al., 1975; 1977; Sansoni and Kracke, 1968) particularly suited for mild ashing of large amounts of soft tissues without loss of volatile elements. It can be easily adapted to routine semi-automation with the added advantage of working with dilute aqueous solutions (Sansoni et al., 1971a,b). The main disadvantage of this method is the inability to ash fat completely. Trace element losses by adsorption on the container walls or on the undecomposed fat, have also to be controlled carefully (Sansoni et al., 1988).

The digestion of biological matter in the vapour phase using a number of reagents, e.g. HNO_3, (HNO_3 + HCO_4), N_2O_4 (Franko and Kosta, 1986) and ozone, appears attractive especially when incomplete destruction of the organic matter can be tolerated. A device for acid vapour decomposition of biological tissue under pressure has been found to be effective (Klitenick et al., 1983).

Wet digestion of biological tissues must be carried out under stringent conditions when the measurement technique to be employed is affected by presence of the organic component (Adeloju et al., 1984). The use of pressure ashing in association with $HClO_4$ (May and Stoeppler, 1984) and also H_2SO_4 (Hasse and Schrammel, 1983; Schrammel et

al., 1987) is suitable for digesting biological tissue prior to elemental analysis by anodic stripping voltammetry.

Body fluids and excreta

Human biological materials in this group comprise whole blood, serum, plasma, cerebro-spinal fluids, erythrocytes, milk, sweat, saliva, urine and faecal samples. The most common analytical method employed for these samples to date has been AAS. Other competitive techniques including NAA, ASV ICP-AES and ICP-MS have, however, been gaining rapidly in popularity. The collection and preparation of body fluids involves careful control in order to avoid hemolysis and consequent cross contamination. The preparation of neonatal blood is fraught with additional problems because of the very small sample size available for investigation. The complete separation of RBC cells from trapped serum can also pose problems. The use of a syringe and needle to transfer the centrifuged cells has been recommended (Sansoni and Iyenger, 1978).

Blood, serum and plasma

The analysis of these biological specimens should be completed as soon as possible and certainly within a few days. Alternatively the samples need to be frozen, lyophilised or pretreated (Stoeppler et al., 1979). However, the losses of trace elements during drying (Behne and Matamba, 1975; Iyenger et al., 1978) and the possibility of contamination from the storage vessels should not be overlooked (Meranger et al., 1981; Williams, 1979).

Complete ashing of organic matter is not essential when determining these biological specimens by AAS unless a preconcentration step is involved. Most body fluids can be aspirated into the flame/plasma directly or after dilution. The specimens can also be introduced into the carbon cup directly for GF-AAS measurements or after matrix modification. A number of matrix modifiers, e.g. ammonium nitrate, ammonium phosphate, lanthanum nitrate, etc., have been used. The deproteinisation of these samples is also helpful (Berman, 1980). A well balanced combination of different approaches is usually helpful.

Enzymatic decomposition of whole blood was found useful prior to its introduction into the graphite furnace (Christensen and Pedersen, 1986).

The determination of certain trace elements which are bound to the matrix can be affected seriously by the organic matter. In such cases, it is necessary to ash the specimens using dry or wet ashing procedures. The ashing in a stream of oxygen (Knapp et al., 1981) is very effective when volatile elements are to be determined. In the authors' laboratory the use of LTA for ashing of blood and serum samples has been in routine use, and found to effectively destroy organic matter without loss of the volatile elements Cd, Pb, As and also to yield low blanks (Sansoni et al., 1988). It is also useful for preconcentrating certain elements prior to their analysis by PIXE (Gocklowski et al., 1984).

Wet digestion of blood and serum can be carried out at normal as well as higher pressures (Knapp, 1988) using common mineral acids without or in combination with

perchloric acid. Several wet digestion procedures for complete removal of organic matter from small blood samples have been studied. It was found that the digestion under pressure was faster and further produced lower blank values (Oehme and Lund, 1979). The procedure is to be highly recommended when voltammetric procedures are being employed for analytical measurements. Decomposition of whole blood in a microwave oven has been employed for estimating a number of elements by AAS (Burguera et al., 1986)

Perchloric acid-free digestions have been usually preferred by a number of investigators (Frank and Reynolds, 1976) in view of the hazards involved in the use of perchloric acid (Gorsuch, 1970).

The irradiation of ashed solutions using ultraviolet radiation has been proposed to get rid of any residual organic matter while carrying out determinations by voltammetric techniques (Nürnberg, 1981).

Sweat, saliva

These samples involve technical problems during collection but are otherwise usually easy to handle and prepare for trace element analysis. The sweat samples are centrifuged or filtered so as to separate the accompanying cell tissue. The samples usually contain a high salt content but can often be directly introduced in the flame or graphite furnace after dilution for measuring a number of minor and trace elements.

The organic matter can be destroyed using procedures similar to those employed for serum. The toxic trace elements are generally present as volatile species and the digestion step should take this aspect into account.

Cerebro-spinal fluid (CSF)

The estimation of various minor (Ca, Mg) and trace elements (Al, Cu, Fe, Mn, Rb, Se and Zn) is usually required on samples of CSF obtained from neurological patients. The levels of trace elements being extremely low, a strict control of sample contamination is absolutely necessary. The needles used for lumbar puncture can cause contamination from iron, nickel and chromium (Lakomaa,1980) and this should not be overlooked.

The samples can be introduced into the flame or the graphite furnace directly or after dilution with deionised water for measurement of several elements by atomic absorption techniques (Panday et al., 1982). Since the specimens usually have low protein content, external ashing and separation steps are not a serious problem. The samples of CSF need be treated the same way as serum if removal of organic matter is necessary.

Milk

The human milk samples need to be deep frozen or lyophilised after collection, and any sample contamination avoided in view of the low levels of many trace elements in these specimens. Many elements (Cu, Fe, Zn, Mn, Sr) can be determined in milk spe-

cimens directly using AAS (flame and GF-AAS) without removal of the protein or some-times after deproteinisation with mineral acids. The samples contain a high and generally variable amount of fat which is not easily destroyed by most of the available wet digestion procedures.

The use of dry ashing procedures for milk specimens is usually preferred in view of the simplicity and the possibility of destroying fat completely. The dry ash can be dissolved in mineral acids directly or after a more vigorous treatment with strong oxidising agents such as hydrogen peroxide. Combustion in the oxygen stream has been found very effective and a number of elements in milk have been determined (Byrne et al.,1979). The dry ashing of milk samples in the vapour phase using nitrogen dioxide was also found useful in estimating a number of volatile and other species (Franko and Kosta, 1986).

Wet ashing under pressure is useful for mineralising the milk specimens for measurement by most instrumental techniques. Its effectiveness for voltammetric techniques has been demonstrated using a mixture of nitric, perchloric and sulphuric acid (Hasse and Schramel, 1983). The use of strong oxidants, e.g. vanadium pentoxide and perchloric acid, should be made with caution and preferably after preliminary ashing of the samples in a furnace or prolonged treatment with concentrated nitric acid.

Urine and faeces

Methods commonly used for determining the elemental content of these biota samples include AAS, NAA, DPASV and ICP-AES. A pretreatment step is almost always necessary. The samples of urine should be acidified with a mineral acid or acetic acid for temporary preservation. The samples should, however, be freeze-dried or lyophilised for long term storage. It is possible to determine a number of elements in urine by diluting the samples with deionised water and aspiration into a flame or plasma.

However, destruction of the organic matter is often desirable for a release of the bound metal atoms. Dry ashing at a temperature of 400°C suffices in obtaining white residues if a few drops of nitric or hydrochloric acid are added after preliminary ashing at a lower temperature. The question of the loss of volatile elements is still unsolved since many elements are present in a complex form. A preliminary decomposition with a mineral acid in an autoclave should preferably be carried out to mineralise the volatile elements in urine.

The freeze dried or lyophilised samples of excreta can be digested at low temperatures (170–210°C) for 15–20 min with a minimal acid mixture of nitric and perchloric (1:2). Though this procedure can not destroy the organic matter completely, it should be at least as effective as the mineral acid extraction procedures described for excreta samples (Riner et al., 1974). Maurer (1977) also found better results by extracting the specimens with a mixture of nitric (65%), hydrochloric acid (25%) and water (3:27:20) compared to those obtained by dry ashing.

Wet digestion of excreta samples using Fenton's reagent has been found extremely efficient and provides clear digests within 6–8 h. The solution is well suited for estimating a

large number of elements including volatile species by AAS, GF-AAS and ICP techniques (Sansoni et al., 1988).

SUGGESTED PROCEDURES FOR SAMPLE TREATMENT OF HUMAN BIOLOGICAL MATERIALS

Suggested sample treatment methods which could be satisfactorily employed for specific human biological material prior to trace element determinations by different instrumental techniques are outlined below:

Teeth

Pre-treatment

Scrape the extracted teeth from adherent tissue using either a quartz or a teflon coated tool. Clean the samples with a brush using detergents and an abrasive, e.g. alumina or pumice, slurry. Wash the samples thoroughly with deionised water and dry at 90–100°C for a few hours. Separate enamel from dentin mechanically by chipping the soft enamel (Retief et al., 1974) or by slicing with the help of a high speed saw (Losee et al., 1974). Rinse the portions of enamel and dentin in double distilled acetone, and dry. Cool the dried samples in liquid nitrogen and grind to a fine powder in a teflon ball mill using the brittle fracture technique (Iyenger, 1976).

Sample Dissolution

The ground samples of enamel and dentin can often be used directly for measurements by NAA, GF-AAS and other techniques which employ solid sampling. It is, however, necessary to dissolve the samples for measurements by other instrumental analytical techniques. Enamel contains very low amount of organic matter and can be dissolved using a single mineral acid. It is usually necessary to eliminate the organic matter from dentin when various dry or wet ashing techniques can be used.

Enamel

Accurately weigh the sample of enamel and transfer to a thoroughly cleaned Erlenmeyer flask. Add 3 mL of 6N nitric or hydrochloric acid and warm slowly at a low heat till a clear solution is obtained. Boil for a few minutes, cool and make up to volume with ultrapure water.

Dentin

The dry ashing of dentin samples can be carried out in a muffle furnace at a temperature of 450°C. However, it is best to ash the samples in a low temperature oxygen plasma when volatile elements are to be determined in these samples. The following procedure can be recommended:

Accurately weigh 0.1 g of the sample in a thoroughly cleaned quartz dish and add 1mL of 6N nitric acid. Evaporate to dryness at 60–80°C, transfer the dish to the low temperature ashing equipment and ash for 12–18 h at a power of 250–300 watts. Treat the residue with 5 mL of 3N nitric acid and heat on a water bath for 10 min. Cool and make up the contents to volume in ultrapure water.

For wet ashing of the dentin, transfer the weighed sample to a Kjeldahl flask and treat with 3 mL of 2:1 mixture of suprapure nitric acid (65% v/v) and perchloric acid (63% v/v). Digest slowly at a low heat initially and then raise the temperature to 180°C till a clear solution is obtained and the white fumes are given off. Cool and dilute the contents to volume with ultra-pure water. The solution can be used for determination of major, minor and trace elements including iron, lead, manganese, zinc, tin, barium, cadmium, copper, vanadium, strontium and the rare earths using F-AAS, F-AES, and ICP methods.

The digestion can be accelerated using a pressure digestion system (Knapp, 1988). 0.1 g of the sample is treated with 1 mL suprapure nitric acid (65% v/v) and 0.2 mL perchloric acid (63% v/v) and heated at 100 bar to 320°C for 4 h. The contents are cooled and diluted with ultrapure water to volume.

The wet ashing of dentin using a microwave oven is also very efficient and rapid. Weigh 0.1 g of the sample and moisten with 2 mL each of 6N hydrochloric acid and suprapure nitric acid (65% v/v). Heat the mixture at low power for 5 min. Cool, add 2 mL of hydrogen peroxide and heat at a higher power of 600 watts for 5–10 min until a clear solution is obtained. Cool and make upto volume with ultrapure water.

Bone

Pre-treatment

Scrape the freshly collected samples of bone free of soft tissue using a quartz chisel or, alternatively, a scalpel made of titanium. Split the bone with a hydrofluoric acid washed bone chisel. Thoroughly wash both the halves with a powerful water stream to remove all the soft material. Transfer the bone samples to a boiling water bath and let boil for 2 h. Wash with ultrapure water and dry overnight in an air oven at 105–110°C. Cool first in a dessicator and then under liquid nitrogen. Powder the specimens using the brittle fracture technique. The powdered samples can be directly used for trace element analysis by NAA, spectrographic and certain mass-spectrometric methods. The dissolution/digestion of bone samples can be carried out using dry ashing or wet ashing procedures outlined below.

Dry ashing in a muffle furnace

Weigh the powdered sample of bone (0.5–2.0 g) in a quartz crucible and moisten with 6N nitric acid. Evaporate to dryness at a temperature of 90–105°C and transfer to a muffle furnace heated to 450°C. Ash for 6–8 h. Cool and treat the residue with a few drops of 6N nitric acid and 2ml hydrogen peroxide. Evaporate to dryness and ash at a temperature of

500–550°C for 12–16 h to a white ash. Transfer the crucible containing the ash to a dessicator, cool and record the ash weight. Grind the ash in an agate mortar. Dissolve portions (0.1–0.2 g) of ash in 6M HNO_3 and make upto volume with ultrapure water. The solution can be used for estimating Al, Sr, Cu, Zn, Mn, Fe, Pb, but not Cd, As, Bi, Sb and Hg. If the latter elements are to be determined, it is recommended that low temperature ashing (LTA) in the oxygen plasma be employed.

Low temperature oxygen plasma ashing of bone

Weigh 0.2 g of the powdered sample in a quartz dish and moisten with 1 mL of 6N nitric acid. Evaporate on a hot plate slowly to dryness and transfer the dish to the plasma asher. Ash for 48 h in the LTA apparatus at a power of 300 W (maximal temperature 200°C). Dissolve the ash in 1 mL of HNO_3 (65%). Add 0.2 mL $HClO_4$ (70%) and heat slowly to white fumes. Add a few drops of hydrogen peroxide (30% v/v), boil for 15 min and make upto volume with ultrapure water.

Wet digestion of bone

Weigh 0.2 g of powdered bone sample into a vycor crucible or a quartz Erlen-meyer flask. Add 5 mL of suprapure conc. HNO_3 (65% v/v) and warm on a hot water both for 4h. Add 2 mL of $HClO_4$ (70% v/v) and digest to a clear solution. Boil to white fumes. Add dropwise 2 mL of hydrogen peroxide (30% v/v). Make up to volume with ultrapure water. The use of perchloric acid can be avoided and the digestion accelerated if a microwave digestion system or a high pressure asher of Knapp type is employed (see dentin).

Urinary / Salivary calculi

Grind the calculi in a teflon vibrating mill or in an agate mortar for eliminating chemical inhomogeneity of the samples. The finely powdered material can be used for direct solid sampling in GF-AAS (Struebel et al., 1987) or for neutron activation analysis (McConville, 1974). The wet digestion of the human stone samples may be carried out as below.

Fusion

Weigh the sample in a nickel crucible and mix with six fold weight of the fusion mixture (5 parts of K_2CO_3 + 4 parts Na_2CO_3). Fuse the mixture for 10 min at red heat. Cool, add 5 mL of deionised water and allow to stand for 60 min. Slowly heat to dissolve the contents completely and transfer to a volumetric flask. Rinse and make upto volume with ultrapure water. The solution can be used for measuring Si, Al and other refractory elements (Burr, 1976).

Wet digestion using mineral acid

Weigh 0.05–0.1 g of the ground sample and moisten with 1 mL of 3N HNO_3. Heat to dryness at low heat. Add 1 mL of suprapure HNO_3 (65% v/v), 0.5 mL of $HClO_4$ and boil till fuming stops. Make up to volume with deionised water. The use of a microwave oven should be extremely effective for these specimens and is to be preferred.

Hair

Washing of hair

Place approximately 1–2 g of the hair sample in a teflon beaker, add 100 mL of acetone and stir with a magnetic stirrer for 15 min. Rinse with deionised water and decant. Now add 50 ml of di-ethyl ether and repeat the procedure. Decant, wash with copious flow of distilled water and finally rinse with ultrapure water. Dry the samples in a dust free environment.

Homogenisation

Place the washed and dried hair into a tightly capped teflon vessel containing a teflon coated ball. Cool the vessel in liquid nitrogen and vibrate for a few minutes in a micro-ball mill. Repeat the procedure if neccessary to pulverise the whole sample. Mix the pulverised sample thoroughly in order to obtain an homogeneous sample.

The powdered and homogenised sample can be used directly for trace element measurements by NAA, spectrographic methods and often also for direct solid sampling in GF-AAS. The digestion and ashing of hair can be carried out as below.

Dissolution

The dissolution with a tissue solubiliser (e.g. TAAH) or a mineral acid is often sufficient for flame and GF-AAS techniques for a number of elements. Weigh 0.1 g of the sample in a Kjeldahl flask. Add 3 mL of suprapure conc. HNO_3 (65% v/v) or a tissue solubiliser, e.g. TMAH solution. Gently warm till the sample is dissolved. Dilute with deionised water or a suitable solvent. The solution can be used for a number of elements including Cu, Zn, Mn, Hg, Cr, Cd, Pb.

However, complete removal of the organic components of hair, entails dry or wet ashing of the sample and can be carried out as below.

Dry ashing in a muffle furnace

Weigh 0.1 g of the hair sample in a quartz dish, moisten with 0.2 mL of 6 N HNO_3 and dry on a hot plate. Transfer the dish to a muffle furnace and ash for 12 h at 400°C. Cool and add 5 drops of suprapure HNO_3. Evaporate to dryness and return to the furnace and

ash for 4 h again at 450°C. Cool and dissolve the residual ash in 2mL of 6 N HNO_3 and make up to volume in ultrapure water.

Other dry ashing procedures

Follow the procedure as described for bone for low temperature ashing in the oxygen plasma (LTA).

The technique of ashing in the oxygen stream using a cold finger is similar to that described for milk (see section Milk below).

Wet digestion

Weigh 0.1–0.2 g of the sample into an Erlenmeyer flask. Add 2 mL of suprapure HNO_3 (65% v/v) and 1 mL of $HClO_4$ (70% v/v). Digest for 2 h on a hot plate to a clear solution. Alternatively, digest in a microwave oven for 10 min at low power and add 2 mL of H_2O_2. Heat at full power for nearly 10 min. Cool the colorless solution and make up to volume with ultrapure water.

The sample solutions obtained can be used for determination of most elements by FAAS, GF-AAS, FES, ICP-AES, ICP-MS and voltammetric techniques.

Nails

Washing of nail samples

Place the collected nail samples in a beaker and add a non-ionic detergent, e.g. carbon tetrachloride or acetone. Transfer the beaker to an ultrasonic cleaner for 15 min. Swirl the beaker and decant the solvent. Wash repeatedly with deionised water and finally dry in an uncontaminated environment under an infrared lamp.

Dissolution and ashing

The dissolution and ashing of nail specimens can be carried out using the various procedures described in the case of hair samples. Dry ashing in the LTA and wet digestion in the microwave oven are particularly effective in the case of nail specimens for which determination of the volatile elements is usually required.

Milk

Direct dilution

The freshly collected samples of human milk can be diluted with deionised water or a suitable detergent, e.g. Triton X-100. The digests can be used for estimations of several minor elements by F-AAS, FES or ICP techniques. It is possible to separate the protein by

precipitation or effectively remove organic matter by ashing the milk samples using both dry as well as wet ashing methods.

Precipitation of protein with acid

To an accurately weighed sample of milk (0.5–1.0 g) in a centrifuge tube add 1 mL of 6M HCl. Heat the tube on a water bath for 1 h and centrifuge at a speed of 3000 rpm for 5min. Decant and add 2 ml aliquots of 3M HCl, centrifuge again and pool the super-natants. Dilute the supernatants to volume with deionised water.

Wet acid decomposition under pressure

Transfer 2 mL of the fresh milk specimen (or 0.1 g of dry milk powder) into the quartz ampoules of a high pressure ashing device. Add 2 mL of HNO_3 0.5 mL $HClO_4$ and 0.1 mL $H2SO4$. Heat the HPA unit at 160°C for 8–10 h at a pressure of 100 bar. Cool and tranfer the contents to a quartz crucible. Treat with 1 mL H_2O_2 and boil the solution for 15 min. Cool and make up to volume with ultrapure water. The digest can be used for most elements except probably mercury and arsenic, using F-AAS as well as ICP methods.

Cold finger oxygen ashing

When the volatile elements are to be measured in milk specimens, the cold finger ashing technique (Tölg, 1977; Knapp et al., 1981) should be employed. The combustion takes place in pure oxygen in a very small burning chamber through an IR-radiator system. The volatile elements are condensed in a cooling system filled with liquid nitrogen (cooling finger) and can be refluxed with a suitable acid, along with the non-volatile elements in the residue. The following procedure may be employed:

Freeze dry the available milk specimen, take 0.1–0.2 g and press into a pellet (0.7 mm in diameter) using a tungsten carbide press. Weigh the pellet and transfer to the sample holder. To avoid an explosion like incineration, stick a match cord of ash free filter paper between the sample and the holder. Position the holder into the burning chamber. Pump liquid nitrogen and ignite the sample after delivering oxygen tangentially at a rate of 80–100 l/h.

After the burning is complete boil the acid (usually 2 mL suprapure HNO_3 or a mixture of $HNO_3/HClO_4$), in the test tube below and dissolve the analyte elements in the residue as well as the volatile elements in the cool finger. Collect the leachate and make up to volume with deionised water.

Soft tissues

Dry ashing in a muffle furnace

Dry ashing in a muffle furnace is often useful (Soman et al., 1970) for soft tissues although extreme care has to be exercised as regards possible contamination and losses of volatile elements. The following procedure can be recommended :

Freeze or oven dry the tissues to a constant weight and weigh 0.5–1.0 g of the sample in a platinum crucible. Transfer the crucible to a muffle furnace and gradually raise the furnace temperature to 400°C. Ash for 4 h, cool and add 1 mL $Mg(NO_3)_2$ solution (1%) and 0.5 mL of conc. HNO_3. Slowly evaporate on a hot plate at a temperature of 80–90°C. Transfer again to the muffle furnace and ash for 8–12 h at 450°C. Cool and add 1 mL of 3M HNO_3 and make up to volume in ultrapure water.

Low temperature ashing in an oxygen plasma can be carried out in the same way as described for bone after finely powdering the soft tissue using the brittle fracture technique.

Complete wet digestion using mineral acids

Weigh accurately 0.2 g of the freeze dried sample into a quartz Erlenmeyer flask. Add 2 mL of suprapure HNO_3 and slowly digest at 120°C for 2 h. Add 1 mL $HClO_4$ (70%) shaking at frequent intervals and continue heating at an elevated temperature (160°C) to white fumes of the acid. Cool, add dropwise 2 mL of H_2O_2 and let boil for 1 h. Finally cool the contents and make upto volume in ultrapure water. The heating time can be considerably reduced if a microwave heating device is used.

The digests can be used for determinations by most techniques including AAS, AES, ICP-AES, ICP-MS and voltammetric techniques.

Blood, serum, plasma, semen

Dilution

It is often possible to estimate a number of elements in these specimens directly or after dilution with suitable reagents using F-AAS (Panday et al., 1983) GF-AAS or ICP techniques requiring the sample in a solution form.

(a) Dilution with water: The dilution with ultrapure water is very often employed, the dilution factor being determined by the concentration level of the elements to be determined in the specimens. Usually a dilution of the order of 10 is useful in order to minimise the viscosity effects. The solution can be used for measuring a number of elements including Na, K, Ca, Mg, Li, Zn, Fe and Cu by FAAS and FES in the above specimens. A much larger number of elements can be determined using ICP-MS (Sansoni and Panday, 1991).

(b) Dilution with detergents: Transfer 1–2 mL of specimen into a clean vial. Add 1 mL of 2% Triton X-100 (or another suitable diluent) and shake throroughly. Let stand for 6–12h with occasional shaking and dilute to volume.

(c) Precipitation of protein with TCA (trichloro-acetic acid): Transfer 2 mL of specimen into a clean centrifuge tube and add 2 mL of 10% TCA solution. Warm the tube on a hot water bath for 1 h. Centrifuge at high speed (3000–4000 rpm) for 5 mins and transfer the supernatant to a 10 mL volumetric flask. Add 2 mL aliquot of deionised water. Centrifuge again and transfer the supernatant to the flask. Make up to volume with ultrapure water.

Dry ashing

The dry ashing of these specimens can be carried out either in a muffle furnace or in the low temperature asher (LTA) using an oxygen plasma using the procedure given below:

Transfer 2–5 mL of the fresh specimen into a clean quartz dish/crucible and slowly heat to dryness at a temperature of 90–105°C. Alternatively weigh 0.2 g of the freeze dried specimen into a quartz dish. Transfer the dish to the muffle furnace (heated to a temperature of 450°C) or into a low temperature oxygen plasma asher. Ash the sample for 6–8 h, cool, add 0.2 mL of 6N nitric acid and 0.1 mL of 1% $Mg(NO_3)_2$ solution. Again dry on a hot plate (90°C) and transfer to the furnace. Ash to a white colorless residue, cool and dissolve the ash in 2 mL of 1 N nitric acid. Dilute to volume with deionised water.

The micro-dry ashing method proposed by Schönberger et al. (1984) or the cool finger oxygen ashing using Trac-O-mat is useful for these specimens.

Wet ashing under normal pressure

Pipette or weigh the specimen (1–5 g) into a Kjeldahl flask containing some glass beads. Add 3 ml suprapure nitric acid (65% v/v) and let stand for 1–2 h. Boil for 30 min till fumes of nitrogen oxide subside. Add 1 mL of perchloric (70% v/v) acid and continue heating till the solution is colourless. Add dropwise 2 mL of hydrogen peroxide (30% v/v) and continue boiling for 30 min. Cool and make up to volume (10–25 mL) with deionised or ultrapure water.

Wet digestion under high pressure (HPA)

Weigh or pipette 2 mL of the specimen into the quartz vials available for use in the high pressure asher. Add 3 mL nitric acid (suprapure 65% v/v) + 0.2 mL perchloric acid (70%v/v) + 0.2 mL H_2O_2 (30%). Close and heat the chamber to a temperature of 320°C for 4 h. Cool and dilute the contents to 10 mL with ultrapure water.

Sweat/CSF/Saliva

These body fluids can be often introduced directly into the flame/graphite furnace or the inductively coupled plasma. Usually a dilution (five fold) with deionised water suffices for measurement of these specimens by most analytical techniques, because of their much lower organic content.

The specimens can be dry ashed or wet ashed using the procedures described for other body fluids.

Urine/faeces

Preservation

The urine samples are usually treated with a mineral acid e.g. HCl, HNO_3 or acetic acid for temporary preservation. However, for long term storage these human specimens need to be frozen, lyophilised or freeze-dried. Fecal samples should almost always be lyophilised or freeze dried before treatment.

Leaching of trace elements

The samples of urine/faeces need to be processed for releasing trace elements present as the organically bound species. This can be sometimes carried out by treatment of the specimens with a mineral acid or deproteinisation with trichloro-acetic acid (TCA). The usual procedure follows below.

Treat a weighed portion (about 5–10 g) of the specimen with 2-5 mL of suprapure acid and digest at a temperature of 80–90°C for 2 h. Alternatively, add 10% of TCA and digest as above. Centrifuge at a speed of 3000–4000 rpm for 5 min. Decant and transfer the supernatant to a volumetric flask. Add 2 mL of 3N nitric acid to the residue, centrifuge and transfer the solution to the same flask. Repeat the procedure and make up the washings to volume. This digest can be used for determining a number of elements in urine/faecal samples by AAS, FES, ICP-AES and ICP-MS. Complete removal of organic matter necessitates the use of dry or wet ashing procedures as below:

Dry ashing

Transfer a weighed quantity of the specimen (10–50 g) into a quartz dish or crucible. Slowly evaporate at a low heat (80–105°C) in case the specimen is not already available in dry state. Transfer the crucible to a muffle furnace (or LTA). Ash at a temperature of 350°C for 4 h. Cool and add 2 mL of 6N nitric acid followed by 1 mL of 2% magnesium nitrate solution (only for classical ashing in the muffle furnace). Evaporate to dryness at a temperature of 80–90°C and transfer to the furnace. Ash for 6–8 h at an elevated temperature of 450°C. Dissolve the residual colourless ash in 2 mL of 3N nitric acid and dilute to volume with ultrapure water. Low temperature ashing in the oxygen plasma is to be

preferred when volatile trace elements are to be determined. The procedure used for bone (cf. Low temperature oxygen plasma ashing of bone) is quite effective for these samples.

Wet ashing using mineral acid mixture

Weigh and transfer the specimen to an Erlenmeyer flask and add 5 mL of suprapure nitric acid (65% v/v). Digest on a water bath slowly so that excessive foaming does not occur. After the initial reaction subsides, add 1 mL of perchloric acid dropwise and boil the contents to white fumes. Now add 2 mL of hydrogen peroxide and boil to a colourless solution. Cool and make up to volume with ultrapure water.

Low temperature wet ashing with Fenton's reagent

Transfer a weighed aliquot of the specimen (10–200 g) in an open beaker or an apparatus specially constructed for the purpose. Add 10 ml of 3N nitric acid and boil at 105°C for 2–3 h. Add dropwise 30% H_2O_2 preferably with a peristaltic pump, and continue boiling till a colourless digest is obtained. This can be used for determining almost all elements by various techniques requiring the sample in a solution form.

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Chapter 3

Graphite furnace AAS*

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INTRODUCTION

The concept of analytical furnace AAS was proposed by L'vov just a few years after Walsh proposed flame AAS. The first paper appeared in 1959 and the technique became commercial in 1969. However, the commercial instruments took the easy route of adapting then available flame AAS instruments by replacing the flame with a graphite furnace. This was easy and convenient to do but it led to a decade of poor analytical performance, as we shall explain. L'vov again took the lead (1978) and showed why the commercial instruments were producing poor analytical data. He made some recommendations, but a clearer understanding gradually evolved over the next few years. The stabilized temperature platform furnace, STPF, is the name we have given to furnace AAS (Slavin et al., 1983) when it is used as closely as possible to the theoretical requirements. It is not a proprietary name or technique, but the analytical equipment must be thoughtfully designed for the requirements of furnace AAS. Some workers call the same technique a constant temperature furnace.

THE STABILIZED TEMPERATURE PLATFORM FURNACE

L'vov's original concept was very simple and elegant. Take a small sample and quickly heat all of it to a particular high temperature that will convert all of it to an atomic vapor. This is done, as illustrated in Fig. 1, by putting the sample within an electric furnace. All of the sample including the analyte will be converted to an atomic vapor and the analyte will absorb a portion of the light from a lamp containing a pure element. In this situation, the integrated absorbance, A_i, at the element resonance line will be proportional to the mass of element in the sample. Everything else that is done is just to achieve these simple conditions.

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* This chapter is dedicated to Professor John Ottaway, who had expected to write it.

W. Slavin
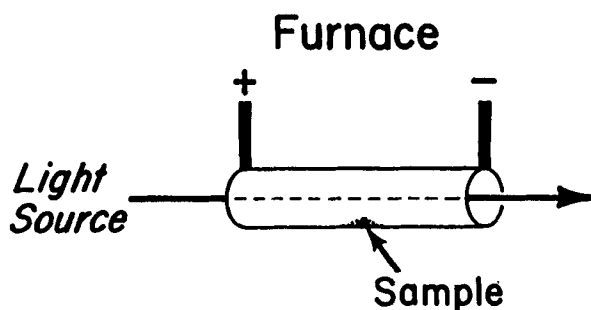
Furnace

Fig. 1. Conceptual design of the L'vov furnace.

The furnace can be thought of as a cell within which the sample vapor is partially confined. If the sample is put on the wall of the furnace, illustrated in Fig. 2, the various materials in the sample will vaporize as the wall heats up. But the temperature and the rate at which the analyte will vaporize depend upon the compounds in which the analyte is present. The L'vov theory requires that the gas phase temperature while the analyte is an atomic vapor be the same for standards and samples. So we cannot tolerate the thermal ambiguity that occurs when we put the sample on the wall of the furnace. Therefore a small platform, Fig. 3, is added within the furnace. The platform is heated by radiation from
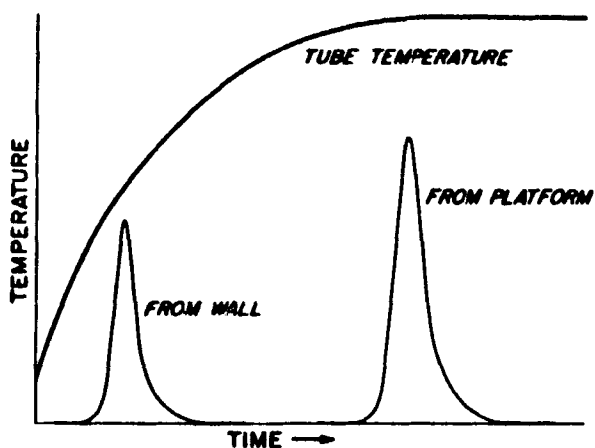
Fig. 2. Volatilization of analyte from the wall of the tube and from a platform. The two absorbance profiles are shown as well as the time-dependent temperature of the tube wall.

GRAPHITE TUBE
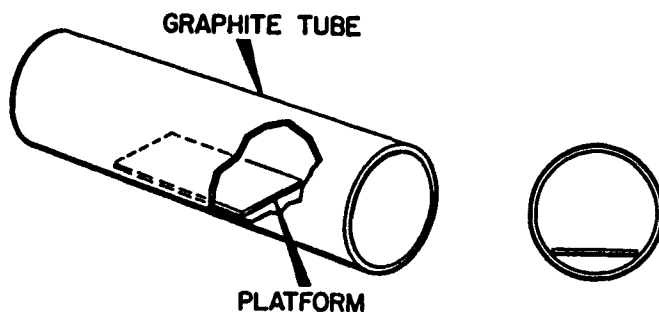
PLATFORM

Fig. 3. Diagram of the L'vov platform and how it fits within the tube.

the furnace walls. This delays vaporization until the walls and the gas within have settled to some quite stable temperature, illustrated also in Fig. 2.

Integration

Integration of the absorbance signal was part of the original L'vov concept. There are many uncontrolled factors that alter the peak shape, therefore the peak absorbance. By now, the literature has numerous examples of the advantage of using integrated absorbance and there is nothing that is more a feature of the STPF technique. In this chapter, the
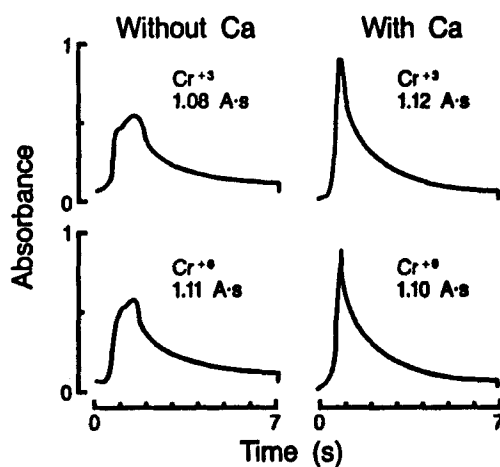
Fig. 4. The effect of the two valence states of Cr on the shape of the absorbance profiles and the effect of Ca on both.

integrated absorbance will be represented by A_i and the peak absorbance by A_p. The units of integrated absorbance are, simply, seconds (s) (Welz, 1992).

When Cr is determined in a Ca matrix, illustrated in Fig. 4 from Slavin et al. (1983), the peak shape is greatly different compared to simple standards. But note that the integrated absorbance signal is the same for both valence states of Cr and independent of the presence of Ca.

However, by itself, integration is not enough; it must be properly accommodated into the instrument system. Small drift in the signal baseline can have a major effect on the integrated signal. This is avoided by taking an automatic zero just as the integration is about to start. We call this a BOC or baseline offset compensation. It is fully automatic on some instruments. Without this function all of the advantages of integrated absorbance are sometimes put aside because, without BOC, the precision is degraded near the detection limit.

Rapid Heating of the Furnace Tube

Early designs of the furnace used just the power to achieve the desired final temperature. However, if more power is applied to achieve the final temperature rapidly, the peaks are higher and narrower. This is especially important for the refractory elements. The maximum power available is used to achieve final temperature quickly, using a photodiode to determine when the final temperature has been reached and triggering a power reduction at that temperature. This has been shown to provide much better performance for metals like V, Ti and Mo.

Fast Digital Electronics

A furnace instrument must use fast signal processing because everything happens quickly and the fast analyte signal must be followed accurately. Older flame AA instruments used slow analog circuits that do not provide usable furnace results.

Matrix Modification

The addition of appropriate chemicals was proposed (Ediger, 1975) which would either stabilize the analyte or alter the properties of the principal matrix, usually making it more volatile. Ediger added NH_4NO_3 to volatilize NaCl as $NaNO_3$ and NH_4Cl, both of which volatilize at temperatures much lower than required for NaCl. He also used Ni to stabilize Se and As, he presumed as the Ni selenide or arsenide. Both of these techniques are still widely used.

In the STPF technique the modifier is usually used to stabilize the analyte to higher temperatures. There are several important advantages to this. We can then char at higher temperatures and reduce the magnitude of the background signal. But more importantly, the analyte can be stabilized on the platform while the STPF conditions are coming to equilibrium.
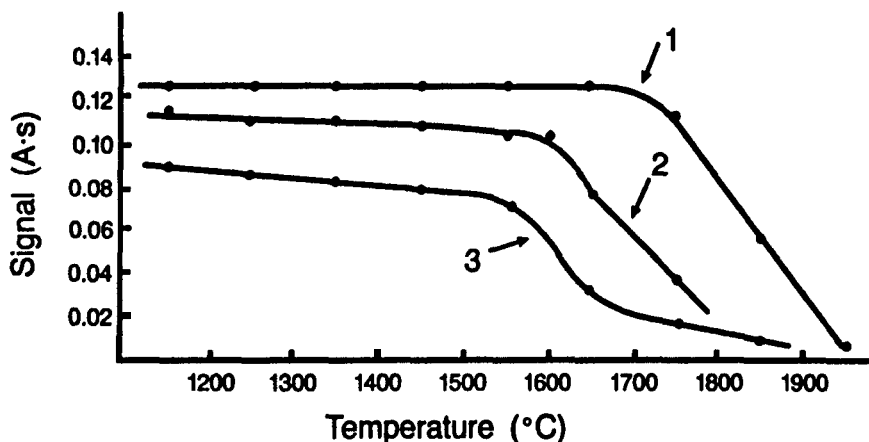
Fig. 5. The effect of $Mg(NO_3)_2$ on the char curves from Cr. Curve 1 is Cr signal in presence of 50 μg of the Mg salt, curve 2 is the Cr signal in diluted urine and curve 3 is the Cr signal without the Mg salt or urine.

We have proposed (Slavin et al., 1982) that $Mg(NO_3)_2$ be used as a modifier to stabilize the more refractory analytes to still higher temperatures. We, and many others, use this compound for Mn, Al, Cr, and for many other metals. An example of its usefulness is shown in Fig. 5 for Cr, from Slavin et al. (1983).

In 1979, a group in China (Shan and Ni, 1979) showed that Pd was an effective modifier for a wide range of elements. Schlemmer and Welz (1986) have tested the combination of Pd and Mg as a modifier and they have been successful with a wide range of analytes. Commercial supplies of Pd as the nitrate are sometimes heavily contaminated with other metals making such salts unsuitable as a modifier for furnace AAS. Palladium salts as the chloride are generally avoided because chlorides are troublesome in furnace AAS. The metal, available as a powder, is most convenient (Schlemmer and Welz, 1986). It dissolves slowly in concentrated HNO_3, and a minimum amount is used. There is usually a small residue that does not go into solution but it is not important to know precisely the amount of Pd in the modifier solution.

Others are finding the Pd modifier useful. We emphasize, however, that it is the combination of all aspects of the STPF technique which produces satisfactory results. The matrix modifiers are often a nuisance if they provide a large blank. But, for many situations, the STPF technique works more reliably with the modifier.

Argon and Gas Stop Conditions

It is important to have two gas streams in the furnace, as shown in the diagram in Fig. 6 of a typical furnace. One stream along the outside of the furnace tube keeps atmospheric oxygen from contact with the hot graphite which otherwise would destroy the tube

Cross – Sectional View –
HGA Graphite Furnace

Internal Gas Flow

External Gas Flow

External Gas Flow

Internal Gas Flow

Window Assy

Graphite Cooling Ring

Light Beam

Graphite Contacts

Graphite Tube

External Gas Flow
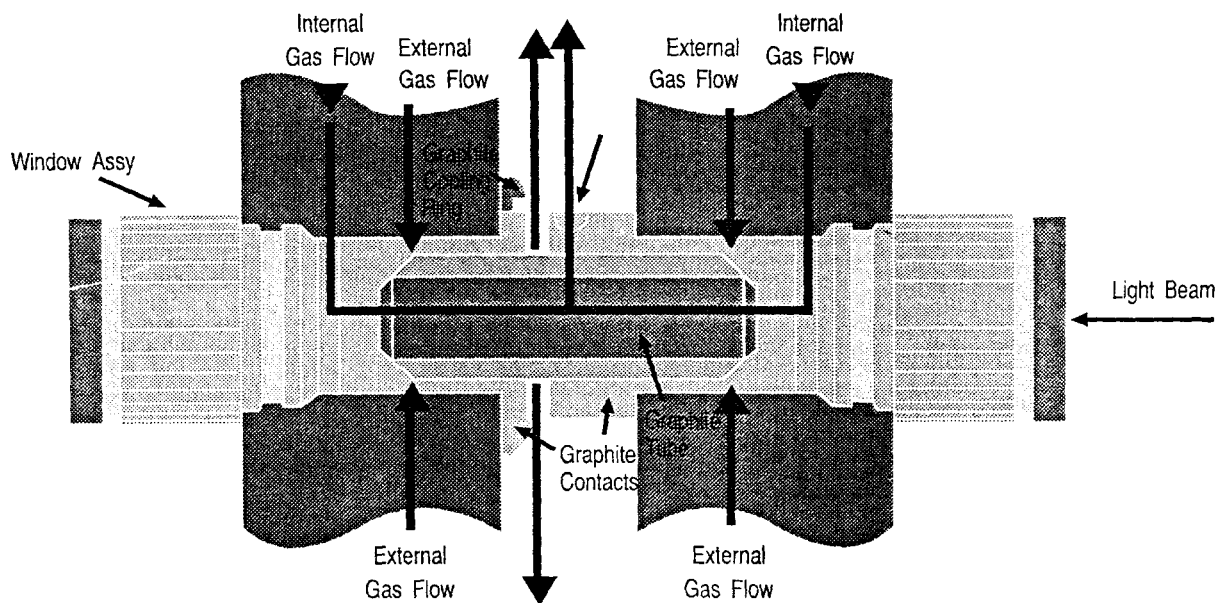
External Gas Flow

Fig. 6. The cross-sectional diagram of the graphite furnace. The two separate gas streams are shown: the internal gas flow which is stopped during atomization and the external flow which protects the outside of the tube from oxidation.

rapidly. The inner gas flow is used to remove the matrix products during the dry and pyrolysis stages. Turning off the internal gas stream during the atomization step provides several benefits. The loss of analyte atoms from the furnace is then controlled entirely by gaseous diffusion. If the gas stream is allowed to flow during the atomization step, cold argon is being blown into the furnace to replace the argon heated by the tube walls. Since the purpose of the STPF technique is to keep the argon fill gas at a constant temperature, gas flow is counterproductive.

Pyrolytically Coated Furnace Tubes

   The walls of the furnace tube are assumed to provide no chemical reactions with the sample. Ordinary graphite at high temperature is porous to many atomic vapors and some analyte is lost through the walls of the furnace if ordinary graphite tubes are used. A layer of dense pyrolytic graphite is deposited at high temperature upon the graphite substrate. This pyrolytic graphite coating is not at all porous. Also, it greatly reduces chemical effects between graphite carbon and the sample. Some people still use un-coated tubes but the analytical performance is thereby degraded.
   The crystal structure of graphite is illustrated in Fig. 7. Six carbon atoms are bound together in a hexagonal array by strong C-C bonding. The flat sheets of carbon in this configuration are bound together in layers. Ordinary graphite is composed of many small domains held together in this way. The chemically active carbon atoms are the end carbons since these have free valence bonds. Pyrolytic graphite differs from ordinary graphite in that these sheets of hexagonally oriented carbon atoms are continuous over the whole surface. Thus there are very few active end carbons. Also, when ordinary graphite is hot, the boundary between the domains enlarges and causes the graphite to be porous to the hot metal vapor; analyte passes through the wall of the hot tube. The
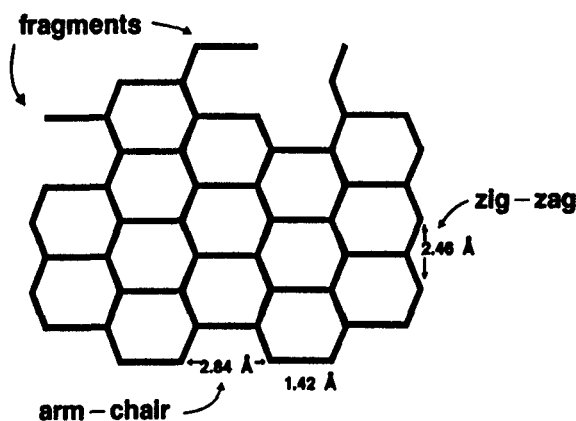
Fig. 7. The structure of graphite showing the planar array of hexagonally bound carbon (from Salmon et al., 1981).

pyrolytic layers prevent that porosity and provide a dense, relatively inactive chamber for the atomic vapor.

Cool-down Procedure

We have recommended what we call a "cool-down" step between the pyrolysis and atomization steps. Its usefulness can be understood from Fig. 8 which shows a diagram of the temperature profile along the length of the tube. The curve marked 2 is the steady state temperature after a few seconds. If the tube is heated from room temperature, the instantaneous heat distribution at the end of the max power step (between 1 and 2 seconds) is shown by curve 3. This is because, with very rapid heating, the only effective method for heat dissipation is radiation, since both conductive and convective heat loss takes finite time. The temperature profile after the char step is shown by curve 1 and, if the temperature is raised directly to the atomization temperature, curve 4 results at the end of max power heating. After a few seconds both curves 3 and 4 decay to the steady state, curve 2, mostly by conduction to the cold ends of the tube. Thus, if the tube is returned to room temperature after the char step, a longer isothermal zone, curve 3 results at the instant the tube reaches atomization temperature. This reduces some of the disadvantageous results of the thermal gradient at the ends of the tube. This technique is particularly useful for the more refractory metals but we now use it routinely for all analyses.
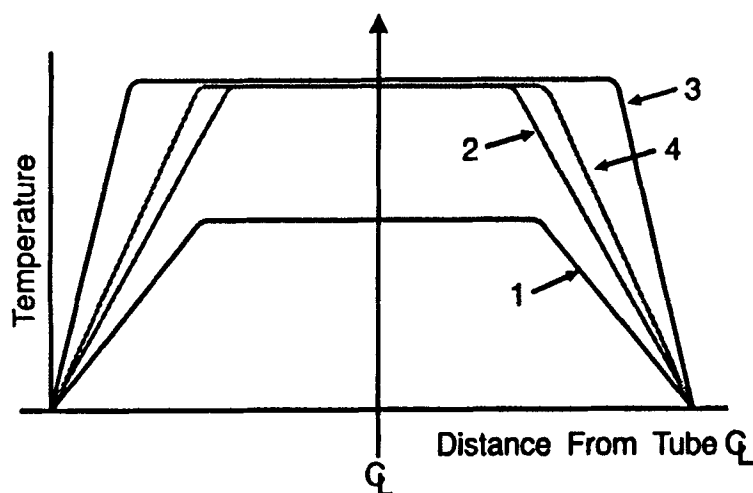
Fig. 8. The temperature distribution within the tube as a function of distance from the center of the tube. See the text for the meaning of each curve.

Background Correction

Almost no real samples can be run without background correction. After an instrument has been in service for some time, the continuum background correction lamps do not always remain in adjustment and correction can introduce errors. This is, beyond doubt, the major advantage of Zeeman correction. Since the same source and optical system are used for both analysis and correction, nothing can go out of adjustment. For this reason Zeeman corrected systems can accommodate much higher backgrounds which produces greater accuracy, as well as lower detection limits, in real samples.

Continuum correction often introduces overcorrection errors for particular combinations of matrix and analyte. Dozens of these errors have been summarized in the literature by Slavin and Carnrick (1988). Fig. 9 shows the problem that arises in tissue samples (fish in this case) when Se is determined. There is a large negative signal caused by phosphate absorption bands in the gaseous phase when a continuum corrector is used. The upper line is background. There is no problem when Zeeman correction is used.

How does one decide if Zeeman correction is necessary for some particular samples? If you are serious about furnace analyses and accurate results are required at low concentrations, a Zeeman corrected system is close to mandatory.

Method of Additions

We almost never use the method of additions (Slavin, 1987). It requires that a preliminary measurement be made in addition to measurements on two or three added aliquots, each producing larger signals than the absorbance of the unspiked sample. Thus, it is always very time-consuming. It is also always less precise than using a working curve prepared from standards (Gardner and Gunn, 1986). It is less precise because each result must be extrapolated back to the horizontal axis. If the additions are not chosen to be large enough, the precision quickly becomes even poorer, again because of the errors introduced by extrapolation. The usual excuse for using the method of additions is that, while slow and imprecise, it will at least correct for unknown errors, thus it is accurate. But that isn't true. The most common errors are background correction errors and these are not corrected by the method of additions. There is no way to know whether the signal from the unspiked sample was caused by analyte or by background (Welz, 1986). Usually it is caused by an unknown mixture of the two.

We construct a working curve of standards plus matrix modifier and we expect to use that curve for all of the samples we must analyze. Sometimes we add approximately the concentration of the major components of the sample into the standard solutions, e.g., NaCl for seawater samples. Usually that is not necessary. In older furnace papers the method of additions was a last resort when all else failed, and it was often used with blind and unwarranted faith.

Deuterium Background Correction

0.2
A

0

Time (sec)

3.0

Zeeman Background Correction

W. Slavin

ZAA 0.2
BG 1.0
A

0

Time (sec)

3.0
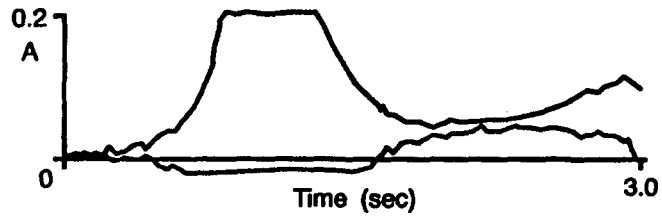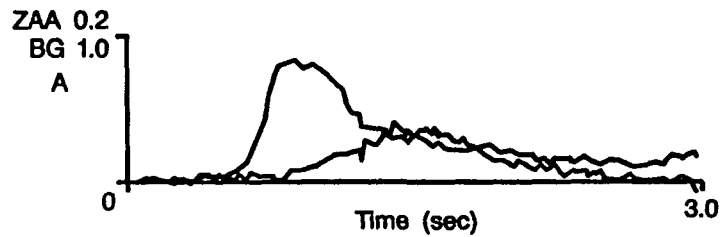
Fig. 9. Comparison of deuterium and Zeeman background correction for the determination of Se in fish tissue (From Welz and Schlemmer, 1986).

TABLE 1

ZEEMAN BACKGROUND CORRECTION

Element	(nm)	Slit (nm)	Site	Modifier	Pretreat (°C)	Atom (°C)
Ag	328.1	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1000	1800
Al	396.2	0.7	P	0.02 mg $Mg(NO_3)_2$	1700	2500
As	193.7	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1300	2100
Au	242.8	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1000	1800
Ba	553.6	0.4	W		1200	2550
Be	234.9	0.7	P	0.05 mg $Mg(NO_3)_2$	1500	2500
Bi	223.1	0.2	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1200	2000
Cd	228.8	0.7	P	0.2 mg PO_4 + 0.01 mg $Mg(NO_3)_2$	900	1600
Co	242.5	0.2	P	0.05 mg $Mg(NO_3)_2$	1400	2500
Cr	357.9	0.7	P	0.05 mg $Mg(NO_3)_2$	1650	2500
Cs	852.1	0.7	P		900	1900
Cu	324.8	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1000	2300
Fe	248.3	0.2	P	0.05 mg $Mg(NO_3)_2$	1400	2400
Ge	265.1	0.2	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1500	2500
Hg	253.7	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	250	1100
In	325.6	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	800	1400
Li	670.8	0.4	P		900	2600
Mg	285.2	0.7	P		900	1700
Mn	279.5	0.2	P	0.05 mg $Mg(NO_3)_2$	1400	2200
Mo	313.3	0.7	W		1800	2650
Ni	232.0	0.2	P		1400	2500
P	213.6	0.7	P	0.02 mg Pd + 0.005 mg $Ca(NO_3)_2$	1350	2650
Pb	283.3	0.7	P	0.2 mg PO_4 + 0.01 mg $Mg(NO_3)_2$	850	1800
Pd	247.6	0.7	P		900	2650
Pt	265.9	0.7	W		1300	2650
Rb	780.0	1.4	P		800	1900
Sb	217.6	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1100	2000
Se	196.0	2.0	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1100	2100
Si	251.6	0.2	P		1400	2650

TABLE 1 (continued)

Element	(nm)	Slit (nm)	Site	Modifier	Pretreat (°C)	Atom (°C)
Sn	286.3	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1400	2300
Sr	460.7	1.4	W		1300	2600
Te	214.3	0.2	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1000	2000
Ti	364.3	0.2	W		1400	2650
Tl	276.8	0.7	P	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$	1000	1600
V	318.4	0.7	W	0.05 mg $Mg(NO_3)_2$	1100	2650
Zn	213.9	0.7	P	0.006 mg $Mg(NO_3)_2$	700	1800

Characteristic Mass

The most important diagnostic we have for furnace AAS is the stable slope of the working curve. The characteristic mass, m_0, has been defined to represent analyte sensitivity. The m_0 is measured in terms of the mass of analyte in pg that will produce an integrated A signal, A_i, equal to 0.0044 s. This measure of sensitivity is analogous to the flame AAS term for sensitivity which is the concentration in mg/L that will produce 1% absorption (0.0044 absorbance). The m_0 values are specific for each analyte and relatively independent of the matrix. Using analytical conditions summarized in Table 1, the characteristic masses are summarized in Table 2. Table 2 also includes detection limits in μg/L. The μg/L detection limits assume a sample aliquot of 100 μL. Variations in characteristic mass of about ± 20% may reflect differences between individual instruments. For an individual instrument, the day-to-day variation should spread less than about 20%. This slope is matrix independent.

Probably the most common explanations of failure to achieve expected m_0 slopes are contamination problems. Some of these are slightly troublesome for flame AA or ICP but the problems are much more severe at the very low levels handled with the graphite furnace. Attempting to achieve a specific m_0 provides the chemist a very powerful means to quality control his laboratory operation.

The most common cause of failure to recover an added spike, outside of contamination, is loss of analyte at the chosen pyrolysis temperature. A pyrolysis study is done on the sample, not on a standard. An unspiked sample is used if the integrated signal level is greater than 0.1 s, otherwise enough analyte is added to produce a signal between 0.2 and 0.4 s. The recommended pyrolysis temperature is used and the temperature is decreased in 200 °C steps from there. Remember that the sample matrix determines the satisfactory pyrolysis temperature.

TABLE 2

ZEEMAN BACKGROUND CORRECTION

Element	m_0 (pg)	Detection Limit $(\mu g/L)^*$
Ag	1.4	0.005
Al	10	0.05
As	15	0.2
Au	10	0.1
Ba	6	0.1
Be	1.0	0.005
Bi	30	0.1
Cd	0.35	0.002
Co	7	0.05
Cr	3.3	0.02
Cs	5	0.03
Cu	8	0.02
Fe	5	0.02
Ga	16	0.07
Ge	30	0.15
Hg	85	0.4
In	12	0.05
Li	1.4	0.02
Mg	0.35	0.004
Mn	2.2	0.01
Mo	9	0.04
Ni	13	0.1
P	3000	
Pb	12	0.05
Pd	24	0.25
Pt	115	0.5
Rb	2.4	0.02
Sb	38	0.2
Se	30	0.2
Si	40	0.3
Sn	23	0.2
Sr.	1.4	0.02
Te	15	0.1
Ti	45	0.4
Tl	17	0.05
V	40	0.2
Zn	0.45	0.01

* Assuming 100 μL aliquot

Summary of the STPF Technique

In summary, the modern furnace technology is the embodiment of the furnace theory developed since the late 1970s. Further improvements can be expected though they will be small by comparison with the differences between the present state of the art and that which predated 1975. The many workers who use antiquated instrumentation and those who are using modern instruments with antiquated methods are foregoing the advantages of the modern graphite furnace. The use of characteristic mass data provides an important quality assurance opportunity. It may eventually become possible to develop absolute furnace methods but, even now, the furnace provides more accurate results in most analytical situations than either the ICP or flame AAS.

ANALYTICAL EXPERIENCE

New Sample

I will outline what we do when a new sample comes into the lab. Standards are prepared to provide signals between 0.1 and 0.3 A and the recommended matrix modifier is prepared as a diluent. We want to start with a simple situation so we dilute the sample until the inorganic solids are less than 1%. Later we'll see if less dilution will also work. A firing using the Cookbook conditions (1985) will provide a rough result. We than add a recovery aliquot to the sample containing about as much analyte as was measured unless that puts the total beyond the analytical range. If the signal in the unspiked sample was less than 0.1 A·s, a spike is added that should produce a signal of about 0.2 A·s. If the recovery in these quick experiments is complete within 5 to 10%, our confidence can be high in achieving accurate results. We look carefully at the stable slope of the working curve, defined as the characteristic mass. The m_0 values are specific for each analyte and are independent of matrix.

If the m_0 is correct, the recovery in the unknown matrix is satisfactory, and a satisfactory pyrolysis temperature has been determined; the rest is detail. We try to use NIST materials to confirm a method and to build confidence. We use NBS 1643, Trace Metals in Water, as a further test of our standards. Also remember that small volumes of solution left in a room environment change concentrations rapidly in both directions because of solvent evaporation and contamination from settling dust.

STPF Successes

By now there are numerous papers in the literature confirming the success of the STPF technique in a wide variety of matrices. The literature is partially summarized in Tables 3 and 4, for various applications. Most of the specific references cited can be found in Slavin (1984). There are still many analytes and matrices that have not been studied and the

TABLE 3

BIOLOGICAL APPLICATIONS

Matrix	Element
Biological materials	Cd, Cr, Ni, Pb
Biological materials	As, Cd, Cr, Se, Pb
Blood	Pb
Blood cells	Cu
Plasma and CSF	Cu
Serum	Se
Serum	Ni
Serum	Al, Co, Cr, Mn, Ni, V
Serum	Cu, Fe, Zn
Serum and urine	Al
Urine	Se
Urine	Cd
Foods	Pb
Infant formula, milk	Pb
Tissue	Zn
Solid hair, nails, skin	Pb, Ni
Animal tissue	Cu, Fe, Se, Ni, Pb, Cd, Mn, Co, Cr, As
Brain tissue	Mn
Fish tissue	As, Cd, Cu, Pb, Se

TABLE 4

ENVIRONMENTAL APPLICATIONS

Matrix	Element
Natural waters	Al, As, Be, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, V
Natural waters	Al, Cd, Co, Cr, Pb, Sn
Wastewater	Cd, Cr, Pb
Seawater	Cd
Seawater	Mn
Seawater	As, Cd, Cr, Mo, Mn, Ni
Marine sediments	Cd, Cr, Co, Cu, Ni, Pb, Be
Marine sediments	As, Be, Cd, Co, Cu, Ni, Pb

literature is increasing rapidly. There are still some difficult situations and we'll touch on these later in the chapter.

Solid Sampling

Most workers come to the analytical situation with a solid, rather than liquid, sample. The graphite furnace probably has a better chance of analyzing such solid samples than any other spectroscopic method. This is because of the long residence time and the constancy of the thermal conditions, but it isn't easy. First of all, it must be possible to get a representative sample in microgram or small milligram quantities. That appears to be feasible for biological tissues but it may not be feasible for geological samples, for example. Many papers have shown success if the representative sample is ground to 10-μm size or smaller. For biological samples there is evidence that particle size need be no smaller than 100 μm.

Schlemmer and Welz (1987) described a solid sampler, shown in Fig. 10, and they have had some success with it. The first panel shows the tool used to introduce the solid sampling cup and an enlarged view of the way the tool grasps the cup. The cup fits within the tube somewhat like a platform. Nevertheless, real samples continue to be troublesome.

The development of solid sampling techniques is an important subject for research activity in the late 1980s. The proceedings of four European colloquia on solid sampling, held in Wetzlar and Jülich, Germany, have been published. Special issues were: Fresenius Z. Anal. Chem., 322 (1985) 653-746; Fresenius Z. Anal. Chem., 326 (1987) 315-418; Fresenius J. Anal. Chem., 337 (1990) 247-319; Fresenius J. Anal. Chem., 342 (1992) 907-956 and R.F.M. Herber (Editor), Pure Appl. Chem., 63 (1991) 1198-1226. The meetings have been organized by Stoeppler and Herber and nearly all of the published papers are in English. Kurfürst developed the Grün Zeeman AA instrument with an emphasis on solid sampling. He summarized the instrumental requirements of solid sampling with the graphite furnace (Kurfürst, 1987).

Getting a representative powdered sample into the furnace remains an important problem. The most promising solution to the problem is by using slurries and many workers are developing this opportunity. Slurries permit simple pipetting into a conventional STP furnace (Slavin et al., 1990; Bendicho and De Loos-Vollebrecht, 1991). The major problem is to keep the slurry homogeneous and this is advantageously done by agitation with an ultrasonic device. This will open up major new opportunities in the next few years. To summarize the current situation and the expectations for solid sampling would require more space than is available for this article.

Fast Analyses

Certainly the major remaining problem with the graphite furnace is its slowness. It takes about 2 min per sample even when the troublesome method of additions is avoided. Dawson et al. (Bahreyni-Toosi et al., 1984) and Halls et al. (1987) have considered procedures to reduce the analytical time per sample to less than 1 minute. Usually this is done by avoiding or reducing the drying step, and by avoiding the pyrolysis step. Of course this increases the background signal and, thus, Zeeman correction is particularly
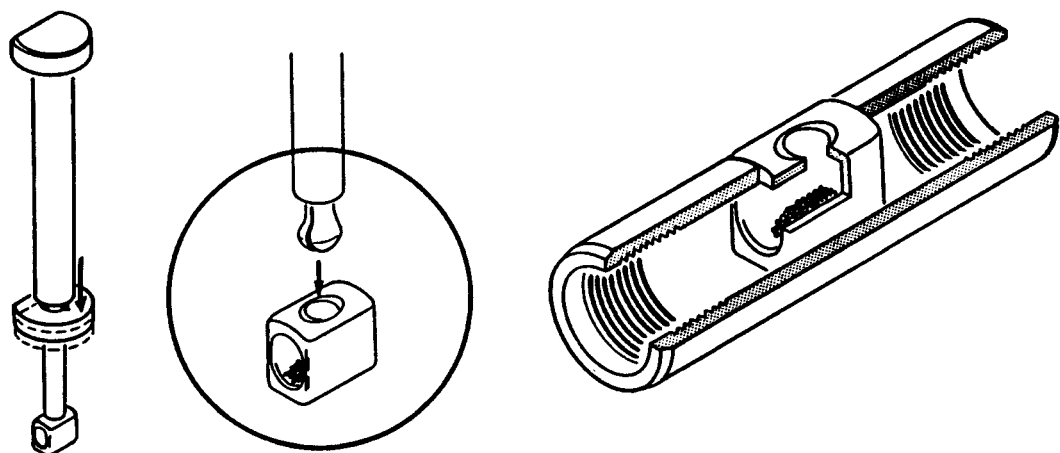
Fig. 10. The solid sampling cup and the insertion tool for the cup. On the left is the tool and cup, in the center the two are disengaged and on the right is an enlarged view of the cup within its special tube.

important for this opportunity. We have avoided the use of a matrix modifier when we avoided the pyrolysis step (Slavin et al., 1989). In this way the actual analytical time was reduced to close to 30 s, although the software on most modern instruments does not yet support analyses at that rate. Many samples can be analyzed at a rate faster than 1 per minute using modern Zeeman corrected instruments and the future will provide still faster analyses. Simultaneous multielement furnace AAS also offers the opportunity of much more rapid furnace analyses if the technique can be reduced to commercial practice in a general and suitable manner.

Some Remaining Problems

Not all elements conform to these happy generalizations. Those elements which are too refractory to determine from the platform, e.g., Ti, V, Mo, Pt, do not conform as well to STPF conditions. We recommend A_i signals for these elements and our experience with V (Manning and Slavin, 1985) indicates that reliable results are found. But more work needs to be done for the refractory metals.

Selenium is one of the most widely determined elements in the Zeeman furnace, but there are still surprises. Some forms of Se are lost as a molecular vapor in the dry and pyrolysis steps and a mixed modifier including $Mg(NO_3)_2$ is recommended. Both Ni and Pd may be used with the Mg.

Barium is a test of instrumental excellence. It is sensitive from the wall of the furnace but it requires a high atomization temperature which, at the long wavelength of the Ba resonance line, often produces a noisy background.

Zeeman Errors

Zeeman correction is not without some potential errors. They are very uncommon and each discovered error warrants a publication. The reason for these rare interferences relates to the shift of very closely spaced lines in the magnetic field causing overlap, or a change in the overlap, of emission and absorption lines. In Fig. 11 from Carnrick et al. (1986) we diagram two situations. In the A panel a sharp matrix line (dotted) is just separated from the analyte line (solid). When the magnetic field is applied (on the right), each of these lines splits into 2 sigma lines each. One of the matrix sigma lines has been shifted under the light source and absorbance results. Since this matrix provided no background in the magnet-off cycle but an absorbance signal in the magnet-on cycle, the difference signal will be <u>overcorrected</u>, and the baseline might fall below zero.

In the B panel the matrix overlaps the analyte emission line with no magnetic field. When the magnetic field is applied, this overlap is reduced and the matrix will have been <u>undercorrected</u> by the Zeeman system. In this case the matrix will cause a signal even when no analyte is present.

This undercorrection situation is exemplified in Fig. 12 with a rarely used Fe line at 271.9 nm which slightly overlaps a Pt line. The dotted Fe absorption is as expected but

MAGNET OFF
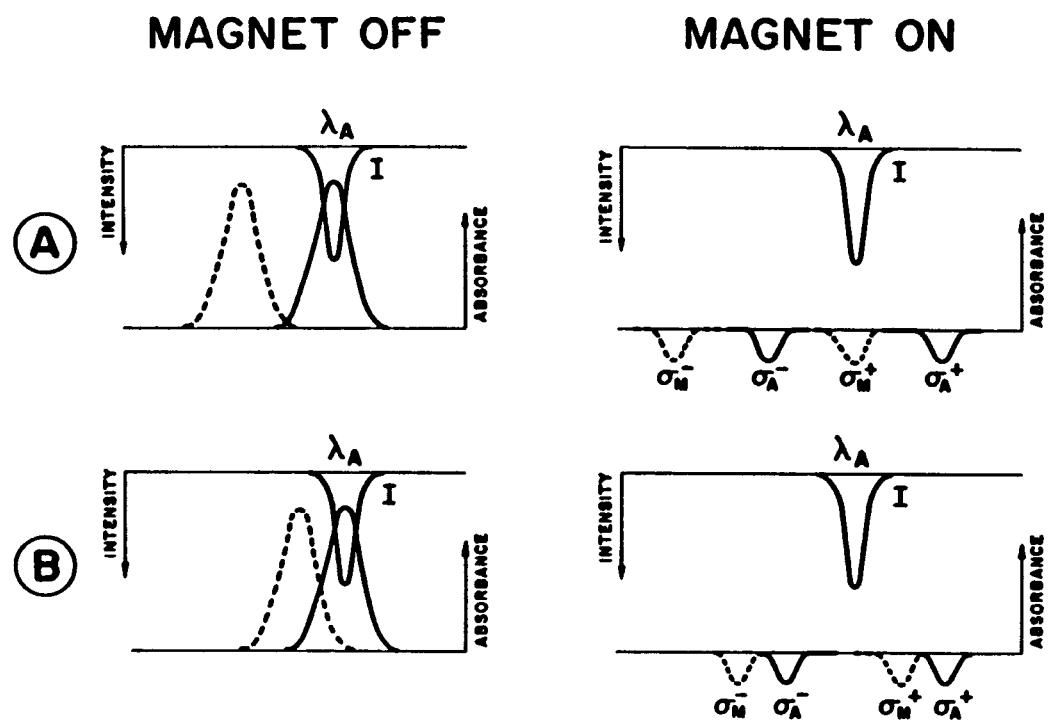
MAGNET ON

Fig. 11. The overlap of two closely spaced absorption lines in the situation (on the left) with the magnet off and (on the right) with the magnet on. See the text for a discussion.

W. Slavin
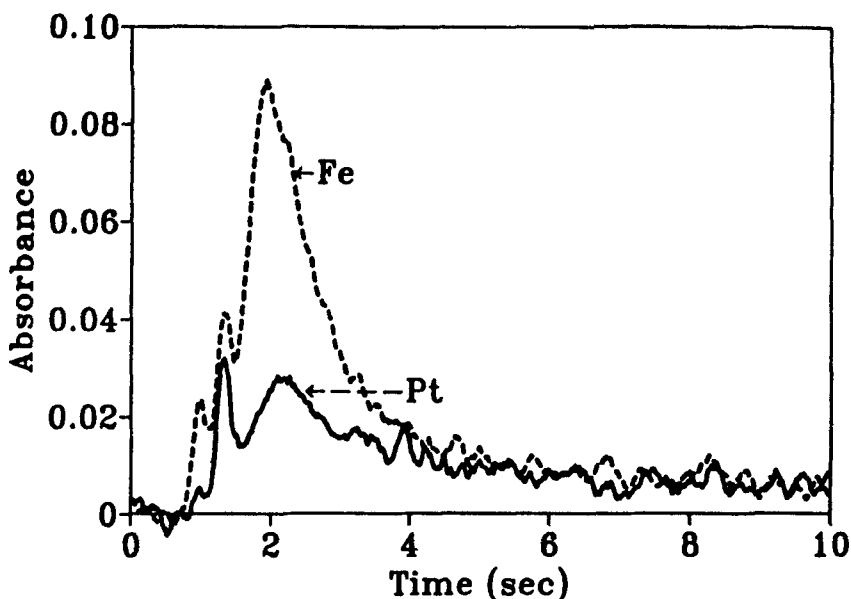
Fig. 12. The undercorrection error generated in a Zeeman magnetic field when Pt is present at the 272-nm line of Fe.

the Pt absorption with the Fe lamp and conditions is an interference since we showed (Carnrick et al., 1986) that there was very little Fe in the Pt solution.

The overcorrection situation is exemplified in Fig. 13, also from Carnrick et al. (1986), due to the sharp absorption bands of PO which almost overlap the very insensitive Cd resonance line at 326.1 nm. But all these examples are more in the nature of science than practicality. Most of the potential problems are at relatively unused wavelengths and some can be avoided by an appropriate choice of experimental conditions.

Elements determined in biological materials in the furnace

The discussion that follows treats separately most of the elements that are determined in biological materials in the graphite furnace. Special attention is paid to the elements that are treated in separate chapters in this book including Al, As, Cd, Cr, Cu, Pb, Hg, Mn, Ni, Se, Tl, V and Zn. However, other elements are discussed that are less frequently determined in biological materials.

Aluminum

The determination of Al in serum by graphite furnace AAS is one of the most published furnace applications because of the role of Al toxicity in dialysis therapy. Proceedings of a symposium on this topic were published (Taylor, 1986) including discussions of methodo-
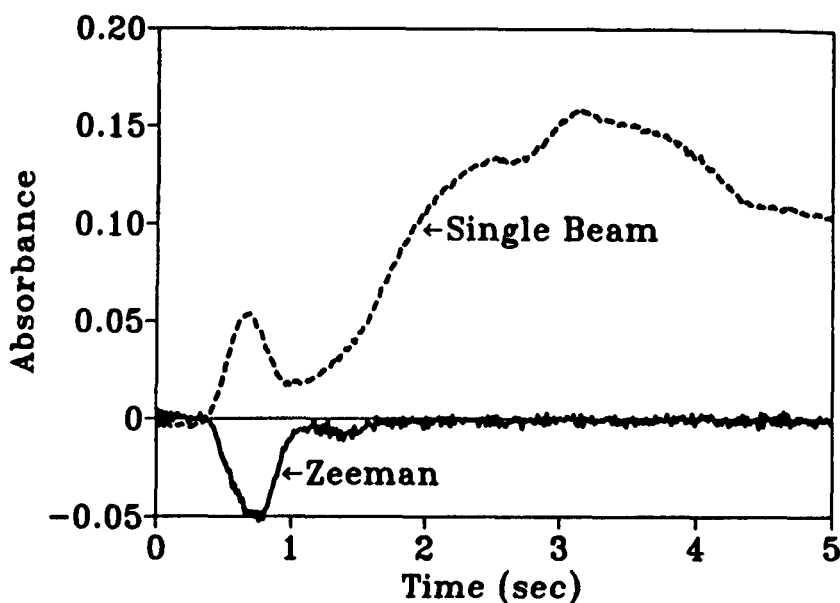
Fig. 13. The overcorrection error generated in a Zeeman magnetic field when phosphate is present at the 326-nm line of Cd.

logy and clinical applications. The causes of perceived analytical problems in some of the recent publications have been assessed (Slavin, 1986). A general problem with the serum Al papers is that most authors have tried to measure normal serum levels (typically less than 10 μg/L) by the same protocol they use for the Al levels that must be monitored in dialysis patients, which are often 100 μg/L or higher. Special care is required to measure normal levels because they are so low. Some of these precautions are not necessary for serum Al levels higher than 50 μg/L. Continuum correction can usually be used because the background is not large, but the precision is distinctly better when Zeeman correction is used.

Pyrolytically coated graphite tubes are important for the Al determination as is the use of a pyrolytic graphite platform. Argon should be used as the purge gas for the Al determination since nitrogen produces a smaller and more variable analytical signal. The Al line at 396.2 nm has been confirmed (Manning and Slavin, 1986) to be preferable to the 309.3-nm line previously used, at least for Zeeman-corrected systems. The sensitivity of the two lines is similar but the 396-nm line provides a much wider analytical range.

Serum samples are diluted with an equal volume of an aqueous solution containing about 2 g/L of Mg(NO$_3$)$_2$, selected to have as little Al contamination as possible. A 10-μL aliquot of this sample is delivered onto the platform. Standards containing 100, 200 and 300 μg/L of Al are diluted with an equal volume of the same Mg(NO$_3$)$_2$ solution that is used for the sample. The Al blank for the Mg(NO$_3$)$_2$ solution is subtracted from each analytical result. This arrangement permits the analytical curve to be plotted in A$_i$ signals

versus μg/L Al in the serum although, of course, the actual concentration of Al in the standards is half that plotted. We use the O_2-ash step at 600 °C but many workers now use air instead of O_2 for this ash step. The baseline offset must be corrected, but the process is automatic in most modern instruments.

Contamination is the most important problem in the serum Al determination (Zief and Mitchell, 1976). Contamination can enter at many points: the metal needles used to collect blood, material leached from the serum collection tubes, impurities in the water, reagents and vessels used, and from air-borne dust. Al is usually present in room dust. Sample handling and separation of serum should be done in sealed containers to the extent possible because dust contamination in the sample preparation stage cannot be distinguished from endogenous Al. When the diluted samples are placed on the table of the autosampler, dust problems can usually be flagged by analyzing two separate serum aliquots from each sample in two separate autosampler cups. Failure to achieve agreement within twice the standard deviation of the method usually indicates a dust problem. Errors arising from dust contamination, like most background correction errors, are positive and additive. They are therefore less likely to cause trouble for high serum levels than for normal levels close to the detection limit.

Normal healthy serum Al levels are surely less than 10 μg/L and are probably less than 1 μg/L. It is probably necessary to use somewhat different analytical conditions to measure normal levels accurately as compared to levels of interest in dialysis patients. The analytical procedure should have a standard deviation approaching 0.2 μg/L which, with an Al detection limit of 5 pg, requires that about 25 μL of serum be in the furnace. A good background correction system is mandatory.

Sample preparation for tissue Al was discussed by Taylor (1986). After dissolution, tissue samples can be treated just as described for serum Al. Urine can be analyzed for Al after a 1 + 4 dilution in the matrix modifier and 0.2% Triton X-100.

Antimony

Antimony can be determined in blood and urine using simple STPF conditions. The presence of large amounts of Fe produces an interference in the determination of Sb at 217.6 nm using continuum correction (Fernandez and Giddings, 1982) and this will probably cause a small error for Sb in blood. The problem is avoided by using Zeeman background correction. Palladium alone, or with $Mg(NO_3)_2$, has been found (Schlemmer and Welz, 1986) to be an effective modifier for Sb. Early furnace papers determining Sb in blood and urine found a variety of interferences that required separation of the matrix by extraction prior to deposition of the extract in the furnace. The use of STPF conditions with Zeeman correction avoids these problems.

Arsenic

Using continuum background correction, phosphate will strongly interfere in the determination of As at the 193.7-nm line (Fernandez and Giddings, 1982). This interference is

not present using Zeeman correction nor does it seem to be present using the less sensitive As line at 197.2 nm. This interference has caused a great deal of trouble in biological samples analyzed by furnace AAS. The presence of Al at concentrations greater than some 10 mg/L will introduce a strong positive error in the As determination at 193.7 nm using continuum background correction (Riley, 1982). This is not usually a problem in biological samples but it may be avoided using Zeeman correction or by accepting twofold poorer sensitivity and using the 197.2-nm line. For toxicology, As has been determined (Eaton and McCutcheon, 1985) using STPF conditions and 20-μL aliquots of whole blood. The blood was diluted 1 + 1 in a solution containing 1% Triton X-100 and the matrix modifier. Modern methods for As use Pd as a matrix modifier. Others have used hydride AAS methods for As, but these methods are not discussed in this chapter.

A good urinary As procedure has been developed by Paschal et al. (1986) using STPF technology and Zeeman background correction. Zeeman correction was judged to be critical to remove matrix interferences. The determination of As in many biological materials requires Zeeman correction but with this technology As becomes a simple determination.

Barium

The determination of Ba is often required for forensic analyses where it is an indicator of gunshot residues. It is a sensitive determination in the furnace providing a detection limit near 1 μg/L. But it is a test of instrumental performance because of the combination of the long resonance wavelength and the high temperature required. Instruments which use background correction with a hydrogen or deuterium lamp only, have little prospect of successful Ba determinations in real matrices but some continuum-corrected instruments offer the option of tungsten lamp correction which, for some situations, is satisfactory. Zeeman correction is preferable.

Beryllium

Beryllium is sensitively determined in the furnace and is suitable for toxicology determinations. It has been determined in urine (Paschal and Bailey, 1986) using STPF conditions and Zeeman background correction with a detection limit of 0.05 μg/L. Urine samples were diluted 1 + 3 in the matrix modifier and 20 μL of sample was added to the platform furnace.

Bismuth

Serum and urine samples have been analyzed for Bi after a 1 + 1 dilution in EDTA (Bertholf and Renoe, 1982). The results would probably have been improved using the STPF conditions and the recommended matrix modifier.

Cadmium

Cadmium is one of the most widely determined metals using the graphite furnace. A direct STPF method for Cd in urine detected less than 0.04 μg/L in the sample (Pruszkowska et al., 1983a). Zeeman correction was necessary because of the large background signals that accompanied the determination. The urine was diluted 1 + 4 in the matrix modifier.

Various biological materials including urine were analyzed for Cd by Yin et al. (1987) using a matrix modifier combining Pd and NH_4NO_3, the STPF conditions and Zeeman correction. Zeeman correction for the Cd determination is particularly important, especially if very small amounts of Cd are to be quantitated. Partly, this is because the background tends to be very large during the determination of Cd because the pyrolysis temperature must be kept quite low. Whole blood was analyzed for Cd by Hoenig (1986) using STPF conditions and air ashing of the sample on the platform.

The World Health Organization sponsored a massive program on the determination of Cd and Pb in kidney tissue and blood and their monograph (Vahter, 1989) should be consulted. Bone samples have also been analyzed for Cd after appropriate dissolution. Cadmium may be determined in solid samples of hair, often for toxicology purposes. If STPF conditions are used, the solid samples can be analyzed against solution standards.

Chromium

The literature before 1980 reported Cr as a difficult determination in biological materials using the graphite furnace. This is chiefly because continuum correction with a deuterium arc provides very poor results ar the long wavelength used for Cr (357.9 nm). By now many commercial instruments use continuum correction with a tungsten lamp which is an important help. Nevertheless, Cr is an element that is much better done with Zeeman correction, especially if the Cr measurement is required close to the detection limit. Pyrolytically coated graphite tubes are particularly useful for Cr. Some workers use mostly STPF conditions for Cr but deposit the sample on the tube wall. Our experience indicates that it is advantageous to use the platform. The pyrolysis conditions are not greatly altered by the $Mg(NO_3)_2$ matrix modifier but small and variable amounts of Cr are lost during the pyrolysis step if the matrix modifier is omitted.

Some workers have reported loss of volatile organic Cr compounds at low temperatures, especially in tissue samples and in plants. Recent papers seem not to be troubled by this problem, probably because of the improved experimental conditions. Some workers have used nitrogen as a purge gas instead of argon. It has been shown that narrow CN bands introduce a continuum correction error at the 357.9-nm line in the presence of nitrogen. Argon should be used. Contamination of plasticware, pipets, etc. is a problem with Cr. Older Eppendorf pipets have stainless steel springs which can introduce Cr contamination.

Serum and blood Cr is an important determination now being done frequently with STPF conditions and Zeeman correction (Schermaier et al., 1985). Serum is diluted 1 + 1

in the matrix modifier and 0.2% Triton X-100. Whole blood is diluted 1 + 3 in the same diluent. Air or oxygen is added during the char step to facilitate the ashing of the organic matrix. About 0.02 μg/L Cr can be detected in the dilutions of either material using 40-μL aliquots in the furnace. Toxic levels of Cr have been found in the plasma of welders using these methods (Morris et al., 1985). Urine has been analyzed for Cr by the same procedures (Slavin et al., 1983). Hair and other tissue can be analyzed for Cr using the same conditions suggested for blood and urine, after dissolution.

Cobalt

Cobalt is generally determined at the 242.5-nm line on Zeeman instruments and at the slightly more sensitive 240.7-nm line on continuum-corrected instruments. It is usually determined on the platform, but wall sampling works reasonably well because Co is a high temperature determination. Pyrolytically coated tubes are important for this determination.

There is very little in the literature using modern furnace methods for Co in biological materials. Kimberly et al. (1987) determined Co in urine using mostly STPF conditions and Zeeman correction, except that the sample was deposited on the wall of the tube. They used 10 μL of a 1 + 1 dilution of urine and found no interferences. They reported a detection limit of 2.6 μg/L Co in the urine. We believe the detection limit can be reduced below 0.5 μg/L if the platform and a somewhat larger sample are used.

By analogy with Cr, a very similar furnace determination, it should be feasible to analyze blood directly for Co in a 1 + 3 dilution of the blood in a mixture of the matrix modifier with Triton X-100. See Cr for more details. The detection limit is about 0.5 μg/L in the dilution, which is not adequate sensitivity to measure normal levels of Co. Increased sensitivity is obtained by ashing a larger aliquot of blood or plasma followed by extraction into an organic solvent which is loaded onto the platform. Several workers have erroneously reported "normal values" for Co in blood using direct methods or extractions from inadequate quantities. Normal values are not reliable by direct furnace methods if they are very close to the detection limit.

Plant and animal tissue have been analyzed for Co after simple dissolution and dilution in the matrix modifier. However, the requirement for measurement at levels lower than can be achieved by direct methods usually calls for concentration by ion exchange or by organic extraction.

Copper

Copper is the only element commonly determined in the graphite furnace that suffers a significant loss of sensitivity (about 50%) when the Zeeman corrector is used. Nevertheless, this loss of sensitivity is more than compensated by the better background correction using the Zeeman effect. The Pd matrix modifier many improve the determination of Cu.

Copper has been determined in plasma, red blood cells, and ocular and cerebrospinal fluids (McGahan and Bito, 1983) using simple 1 + 1 dilution in 1% Triton X-100 and

standards prepared in water. The detection limit was about 0.1 μg/L in the dilution. Many workers have determined Cu in urine with the modern furnace. Milk and infant formula have been analyzed for Cu using simple dilution methods and the STPF technique (Delves, 1986). Delves determined the Cu in protein fractions that were separated from 2 μL of serum by cellulose acetate membrane electrophoresis.

Gold

The determination of Au in biological materials stems from its use in the therapy of rheumatoid arthritis. Papers using the new furnace technology for serum, urine or tissue provide an attractive opportunity. A detection limit of about 1 μg/L is possible by direct procedures, once the sample is in solution. A group in Ottaway's laboratory (Shan et al., 1987) compared wall and platform technology for the determination of Au in whole blood and plasma. It is difficult to decide from their paper which techniques were preferable, partly because the patients were on chrysotherapy and the blood levels were high, permitting 1 + 100 dilution prior to pipetting onto the platform. Serum diluted 1 + 1 and whole blood diluted 1 + 3, using Pd plus Mg as a matrix modifier, and the platform with integrated absorbance signals should provide excellent results.

Iron

While flame AAS is adequate for routine determination of serum Fe, micro methods utilizing the furnace have been developed (Lewis et al., 1984) for pediatrics, etc. Of course, deproteinization is still required, usually with trichloroacetic acid. Because of furnace sensitivity, the serum sample is diluted 1 + 9 in a solution containing the matrix modifier and about 0.2% Triton X-100, and a 10-μL aliquot is deposited on the platform. In the dilution, 100 μg/L of Fe will provide a signal of about 0.4 s using STPF conditions. The same method will apply to Fe in tissue and other biological materials, once the sample is in solution.

Lead

The graphite furnace is increasingly the method of choice for blood Pb. A review of Pb poisoning (Boeckx, 1986) published in 1986 found that 25% of the 95 labs participating in a CDC proficiency test for blood Pb used the furnace and an equal number used the Delves cup. This ratio has shifted rapidly to the furnace. For the STPF blood Pb method (Pruszkowska et al., 1983b), whole blood is diluted 1 + 9 in a solution containing 0.2% Triton X-100 and the matrix modifier. Alternatively, the matrix modifier may be added in a separate aliquot using an autosampler. A 10-μL aliquot is deposited on the platform. The standards are made up with 0.2, 0.4, 0.6 and 0.8 ng of Pb in the same diluent that is used for the samples. Background correction is necessary for reliable blood Pb results but the backgrounds are not large and continuum correction provides good results. Zeeman correction provides better precision.

The blood Pb method has been adapted (Parsons and Slavin, 1993) to the transversely heated and longitudinal Zeeman corrected furnace to gain speed and precision. Whole blood, obtained either by venipuncture or fingerstick, is diluted 1:10 in a matrix modifier solution. The modifier solution contains 0.2% w/v $NH_4H_2PO_4$, 0.5% v/v Triton X-100 and 0.2% HNO_3. Twelve μL of the diluted blood (1.2 μL of blood) are deposited on the platform of the Model 4100ZL furnace. Each cycle is completed in 90 s with a program time of 47 s and this permits the analysis of some 100 samples in duplicate in a typical 7.5 hour day. For screening purposes, singlet determinations can be used, doubling the instrumental capacity. Aqueous standards in the same modifier solution are used to prepare the analytical curve. The standard deviation is typically 0.25 $\mu g/dL$ at low concentrations making the method particularly suitable for the current CDC regulations which demand useful information at 10 $\mu g/dL$. The accuracy of the method, using many different reference materials, was better than 1 $\mu g/dL$ at low concentrations and better than 5% at higher concentrations.

The determination of serum Pb with the furnace is difficult because the serum levels are very low. STPF methods and Zeeman correction are mandatory for serum Pb. If, as has been reported, normal serum Pb is less than 1 $\mu g/L$, it will not be possible to measure these levels with confidence with a direct method. A 20-μL aliquot of a 1 + 1 dilution of serum in the diluent mentioned earlier will provide a detection limit a little lower than 1 $\mu g/L$. For serum levels higher than this, the method can be used with confidence.

Urine Pb down to the 1-$\mu g/L$ level can be measured in 20 μL of a 1 + 1 dilution of urine in the matrix modifier. If a somewhat poorer detection limit is acceptable, a 1 + 3 dilution of urine is more reliably handled by the autosampler. Paschal and Kimberly (1985) used a very similar urine Pb method but altered the conditions to make the method applicable to non-Zeeman corrected instruments.

Tissue Pb, particularly animal tissue, has been widely determined by this simple STPF method once the tissue is in solution. For many biological samples, though not blood, the major problem is caused by inadequate continuum background correction. Stimulated by reported problems in the determination of Pb in infant formula, Andersen (1985) used the simple STPF method stated above and Zeeman background correction. Reliable results were found with good precision. Zeeman correction is particularly valuable for the determination of Pb in complex inorganic matrices that produce large backgrounds.

Lithium

There are very few publications on Li in biological materials with the furnace. Furnace sensitivity for Li is very great and a 20-μL aliquot on the platform provides a detection limit lower than 0.1 $\mu g/L$. Before biological methods can be developed, more work is necessary to optimize the conditions for Li using the STPF technique.

Manganese

Manganese is an ideal STPF analyte. The low levels of Mn that are often found in biological materials suggest that it should be determined in the furnace, usually by direct methods. The major problem with the determination of Mn is the control of contamination, which is discussed in an earlier section. Zeeman correction is particularly useful for Mn because, at the long wavelength used for Mn (279.5 nm) the continuum correctors are not very effective.

Whole blood, serum and blood cells have been analyzed for Mn by many workers using furnace methods, although contamination has probably produced large errors in many of these studies. The normal serum level is close to 1 μg/L, probably lower (Versieck and Cornelis, 1980). We recommend that serum be diluted 1 + 2 in the matrix modifier and 0.2% Triton X-100 and 20-μL aliquots be deposited on the platform. This arrangement yields a detection limit of about 0.1 μg/L. The same methods have been used for urine (Frech et al., 1985).

Tissue samples were analyzed by the same technique after the samples were put into solution. Hair samples were cleaned and decomposed in a miniautoclave and a similar procedure was used on the solutions.

Mercury

Mercury is best determined by the cold vapor atomic absorption method. The instrumental conditions for this determination have been discussed by Welz (1985). The graphite furnace can be used to determine Hg but, because a small sample is taken, the sensitivity is not as favorable as the cold vapor technique.

Molybdenum

Molybdenum is a sensitive (m_0 = 9 pg) furnace determination although relatively few papers have been published on biological applications. It is one of the most refractory metals determined in the furnace and therefore the sample is deposited on the wall. Nevertheless, the other STPF conditions are used, including the use of A_i signals. Plant tissue can be analyzed for Mo (Hoenig et al., 1986) after wet ashing in HNO_3 and H_2O_2. The use of A_i signals is important in obtaining accurate results because many matrix materials alter the absorbance profiles. The transversely heated graphite furnace instrument provides narrow absorption profiles for Mo that are easily integrated.

Nickel

Nickel is easily determined in the furnace from the wall or from the platform. Use of the platform and a matrix modifier is preferred for biological materials. Again, it is important to use A_i signals. The IUPAC reference method for Ni in urine and serum was prepared for the graphite furnace (Brown et al., 1981). They extracted the Ni with APDC into MIBK and

analyzed the organic phase in the furnace. Since 1 + 1 dilutions of urine or serum in 20-μL aliquots on the platform would provide a detection limit in the sample of about 1 μg/L and normal values are reported to be only slightly larger than that (Versieck and Cornelis, 1980), a direct method might not be sufficiently sensitive. Sunderman et al. (1984) determined Ni in whole blood and serum using a protein-free solution of the sample and STPF conditions with Zeeman correction. They reported an 0.1 μg/L detection limit using 50-μL aliquots in the furnace.

Phosphorus

Phosphorus has become an important platform furnace element. It is not as sensitive as most other elements because the resonance lines are not spectroscopically available. But reliable determinations at the ng level can be made in biological materials. It is particularly important to use the platform, matrix modifier and A_i signals. Consult Curtius et al. (1987) for a thorough discussion of biological applications of the STPF method for P.

Selenium

Selenium is probably the furnace determination which most demands Zeeman correction STPF technology. Other methods are slow and prone to manipulative errors at the low concentrations that are typically of interest in biological materials. Nevertheless, the volatility of many Se compounds, especially organoselenium compounds, produces troubles. Both Fe and P cause severe overcorrection errors when Se is determined with continuum correction, making Zeeman correction mandatory for Se in biological materials. There are many papers in the literature that have not used Zeeman correction for Se but they rely on delicate timing of the thermal program so that Se is not volatilized at the same time as the interferent. The paper of Verlinden et al. (1981) on the AAS determination of Se should be consulted.

Whole blood and serum may be analyzed for Se by a 1 + 2 dilution in the combined Pd and magnesium nitrate modifier which includes 1% Triton X-100. A 20-μL aliquot of this will provide a Se detection limit in the sample of about 3 μg/L which is adequate for whole blood and plasma for which normal values range near 100 μg/L.

The determination of Se in urine was reported (Carnrick et al., 1983). High levels of sulfur in urine were found to interfere in the Se determination unless the magnesium nitrate was present. But 1 + 4 dilutions of urine provided reliable results with detection limits below 10 μg/L in the urine. Modern work suggests that Pd may be a preferable modifier for Se (Schlemmer and Welz, 1986).

Silicon

There is some interest in the Si determination in biochemical samples. There is every reason to be optimistic about the potentiality of the furnace for this application but, at present, there are few practical publications. There are many physical-chemical studies in

the literature using pre-STPF technology. We recommend that the platform be used, see Table 1, but we have not looked for an appropriate matrix modifier. The solution detection limit is probably very close to 1 μg/L using a 20-μL sample. Some graphite furnace tubes provide a large Si blank resulting from Si bound within the graphite substrate.

Silver

Silver is sensitively detected in the graphite furnace and is relatively free of interferences when STPF conditions are used. There is very little literature on biochemical applications but there is a growing literature on environmental materials. The experimental conditions for Ag with STPF and Zeeman correction were studied (Manning and Slavin, 1987) and it is recommended that Pd be used as a matrix modifier.

Tellurium

Tellurium is rarely sought in biological materials but it will pose no problems when determined with STPF conditions. Like As and Se, there are problems in the presence of phosphorus if continuum correction is used and Zeeman correction is recommended for biological materials (Fernandez and Giddings, 1982). The preferred modifier is Pd.

Thallium

Thallium is sensitively determined in the furnace which has been used frequently for toxicological problems. The determination is prone to interference in the presence of large amounts of chloride because vapor phase chlorides of Tl are quite stable. The literature prior to the STPF technique showed considerable interferences which are not seen with modern technology.

In general, chloride is avoided in sample preparation for furnace AAS because many metal chlorides are relatively stable as gaseous molecules. But Tl has the highest stability of the metals often determined in the furnace so it is particularly important to minimize the chloride content of samples. The nitrate salt of Pd is the preferred modifier in the presence of several % HNO_3 (Manning and Slavin, 1988). Welz et al. (1988) also studied the STPF conditions for Tl and recommended that the Pd modifier be pyrolyzed onto the platform prior to deposition of the sample. With this arrangement they were able to measure Tl in a seawater matrix.

There are many reports in the literature on the determination of Tl in blood, serum and urine, as well as other biological matrices. See, for instance, Paschal and Bailey (1986) who used STPF methods for the determination of Tl in urine. A detection limit of about 2 μg/L in urine, blood or serum can be achieved if the sample is diluted 1 + 1 or 1 + 2 and 10 or 20 μL of the diluted sample is used in the furnace. For blood samples, Triton X-100 should be added also.

Tin

In many general analytical studies, Sn has proven troublesome in many matrices, particularly in the presence of sulfate. We have found no trouble with STPF conditions (Pruszkowska et al., 1983c). There are few methodology papers on tin in specific biological fluids or tissues but the general procedures are very likely to work well. A dilution of blood or urine of 1 + 3 in a solution of Pd as the nitrate will provide a detection limit of about 4 μg/L in the sample using a 20-μL aliquot on the platform.

Vanadium

Vanadium is a refractory element determined from the wall of the furnace but otherwise using STPF conditions (Manning and Slavin, 1985). Urine diluted in 2% HNO_3 and Triton X-100 was analyzed for V by Paschal and Bailey (1990) using STPF methods with a detection limit of 2 μg/L in the urine. Shuttler (1992) used the transversely heated furnace (the Perkin-Elmer 4100ZL) and, with only dilution of the urine, was able to obtain a detection limit better than 0.5 μg/L.

Zinc

For micro determination of Zn in serum or urine, the furnace is used with STPF conditions. Because of the sensitivity of the furnace Zn method, the determination is very prone to contamination. Work near the furnace detection limit probably requires clean room facilities.

For pediatric or micro serum Zn, serum was diluted 100-fold and a 10-μL sample was deposited in the furnace (Vieira and Hansen, 1981). The STPF technique was still too sensitive. The Zn line at 307.6 nm appears to have about 1000-fold less sensitivity which makes it too insensitive. It would be preferable to dilute the sample 200-fold and use a 5-μL aliquot. Because of the sensitivity, Zn has been determined in serum fractions separated by chromatography (Gardiner et al., 1981). Ten-μL urine samples were analyzed directly (Vieira and Hansen, 1981) in the furnace.

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Atomic absorption spectrometry

Flame AAS

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Atomic absorption methods are the most frequently used for the determination of trace metals in a wide range of materials. This review is written at a point in time when most analysts still view the graphite furnace technique as an accessory to flame atomic absorption. Actually the two techniques are quite separate and independent. This review is thus written with the understanding that atomic absorption is two techniques, flame AAS and furnace AAS. This position will not be defended here but such a defense has been published (Slavin, 1986). Thus we treat the two techniques in separate chapters.

Flame AAS is generally the best analytical technique available for samples that are most easily collected as solutions, if the analyte concentration is above the mg/L range in the solution. Precision of about (RSD) 1% is routinely obtained. Better precision approaching RSD = 0.2% is available if extra care is taken in the preparation of standards and if slightly more time-consuming methods are used. These methods include the preparation of accurate standards made up to bracket a preliminary determination.

When the solution sample is suitably diluted, usually in simple media, the analyte may be quantitated in seconds. Automation is widely and relatively inexpensively available. Atomic spectroscopy is inherently very specific and interferences that will reduce the bias below the level of the precision are few. Those interferences that exist are well characterized and are generally controlled in a routine manner. The net result of all this is that flame AAS should be used in every situation where it is applicable.

There are some exceptions to the generalizations above. The very refractory metals, e.g., W, Ta, etc., are not reduced to an atomic vapor in the flame and are therefore not accessible to flame AAS. In this case ICP is usually preferable. If the analyte concentration approaches the μg/L level or below, the precision becomes much poorer. In such cases other more sensitive methods are often preferable, for example, furnace AAS. If many metals must be determined in each sample, ICP emission spectroscopy is often more rapid, though it is more expensive and it requires a higher level of operator skill than flame AAS.

If the sample is a solid, dissolution is required prior to flame AAS. This slows the process and opens the opportunity for contamination during the various handling steps. It would be very convenient if there were a way to introduce solid samples directly into the flame. Papers have occasionally appeared in the literature but no solid sampling technique for flame AAS has been found to be generally acceptable for biological samples.

Flame AAS is well established and has been discussed exhaustively in many accessible books and reviews (Welz, 1985; Price, 1979). We do not feel that there is a need to discuss here the theory or the general analytical technique.

Mercury and the 'P' block elements are very sensitively determined on flame atomic absorption instruments by separating the element from an appropriate sample solution as a gas which is swept into the monitoring optical system. It is either an atomic vapor or it is reduced to the atomic state and the atomic absorbance is measured. For Hg, this is usually done by adding tin chloride to the solution to be analyzed, which liberates free Hg. Because of its volatility, the Hg passes to the vapor phase and is swept to a cell in the AA spectrometer. For the 'P' block elements, gaseous hydrogen is bubbled through the solution forming a metal hydride which is swept into a quartz cell heated by the AA flame. The hydride is dissociated and the gaseous atoms absorb source radiation providing a sensitive analytical signal. An important advantage of these two techniques is that a relatively large sample, e.g. 1 to 100 mL, is used, providing relative detection limits that are usually better than furnace AAS, certainly much better than can be obtained with flame AAS. Appropriate equipment is provided by most AAS manufacturers as a relatively inexpensive accessory for the flame AA spectrometer. The hydride technique is widely used for As and Se in biological and environmental samples, and is also sometimes used for Sb, Bi and Te. There have been publications discussing hydride methods for Ge, Pb and Sn but these are certainly not yet routine.

Some interferences are removed by the hydride technique because the analyte is physically separated from the remainder of the matrix. But there are interferences in the process of generating the hydride and the variability in the rate of generation. There appears to be some black art in the quartz decomposition cell, and sometimes simple electrical furnaces are used to convert the hydride to the atomic vapor. In the opinion of this author, the furnace technique has fewer interference problems for the 'P' block elements than the hydride technique but the furnace equipment is more expensive and, if the quantity of sample is not limited, the furnace is less sensitive. There is a great deal of historical and practical information on both the cold vapor method for Hg and the hydride method in the AAS book by Welz (1985), who is an experienced authority in these methods.

There are many excellent publication on biological applications using methods of atomic spectroscopy, including flame AAS. Morrison (1976) reviewed the determination of trace metals in biological materials with considerable attention to comparisons between the available analytical techniques. He showed comparative detection limits of the several techniques and the levels found for each essential and toxic metal in serum and other biological matrices. Sample preparation and control of contamination were discussed. Mertz (1981) discussed the status of the essential trace elements in human biology. Each

was separately discussed and its biochemical role was evaluated where it was known. While the paper did not discuss analytical methods, it was a superb review of the nutritional role of metals.

A review of elemental analysis of clinical materials was prepared by Delves (1981). Each essential trace element was treated separately as well as many non-essential trace elements including toxic metals. A compilation of papers on trace metals in medicine and biology was published by Brätter and Schramel (1980). There were several valuable papers on individual metals, on various analytical procedures and on the role of trace elements in human and animal biochemistry. Other volumes of this book provided much valuable information in biochemistry.

Many papers have been published directed to the preparation of biological samples for analysis and to methods for avoidance of contamination. Versieck (1983) discussed errors arising from needles, vacutainers, etc, for Cr, Ni, Cd, Pb, Mn, Cu and other metals. He also discussed errors arising in neutron activation analysis. His emphasis is on problems relating to contamination.

Berman (1980) has written a monograph on toxic metals. She discussed the biological and pathological significance of many trace metals and the various analytical procedures for their determination. The book was arranged by element. Risby (1979) prepared a monograph of a 1977 ACS meeting devoted to trace metals in biochemistry. There was material on nutritional requirements, on Mo in biological material, the role of Zn and Cu in biological systems. The classic book on trace elements in biochemistry is by Bowen (1966) and it has avoided obsolescence because it does not address analytical methods. It discussed terrestrial geochemistry, the role of trace metals, the composition of living matter, etc. Muramatsu and Parr of the IAEA (1985) surveyed the reference materials available for biological analysis. The listing is detailed and complete. It includes analytical levels listed in each material.

Sample preparation for biological samples have been described. Moody (1983) reviewed sample preparation and storage, mostly for biological samples for trace metal analysis. As part of a seminar on biological sampling at the NBS, Parr (1986) discussed sampling and sample preparation for trace metal analysis. He emphasized the problem of contamination and how to avoid it. He recommended methods preferable for particular analytes and, as for most of us, he finds his own field, NAA, most widely applicable. That whole issue of J. of Research, NBS, is worth consulting on biological sampling. In the same issue Versieck warns of the problems associated with the collection and preservation of plasma or serum for trace metal analysis. Again the dominating effect of contamination is emphasized. Sunshine (1975) assembled specific methods for analytical toxicology in 1975. Many of these utilized flame AAS.

The classic book on sample preparation is by Gorsuch (1970). He discussed wet and dry oxidation and fusion. Each element was considered separately. The book should be consulted in a new or difficult situation. The control of contamination in trace analysis has been addressed in a book by Zief and Mitchell (1976). They discussed the life of various standard solutions at the mg/l level at various pH after 24 hours. The losses were great for many ions at high pH, but there were no losses at pH lower than about 2. The same effect

was found for water samples. Sources and extent of contamination were assessed and clean room designs were provided. Container materials were compared. Methods for purifying reagents were described. There is some discussion of methods at the ultratrace level and furnace AAS is included but the material is very old.

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Atomic emission spectrometry

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INTRODUCTION

Modern analytical chemistry, especially for trace analysis, is mainly dependent on physical techniques (Broekaert and Tölg, 1987). For inorganic trace element analysis several different methods exist such as activation analysis, x-ray fluorescence, mass spectrometry, electrochemical methods and the different techniques in atomic spectrometry. There is no universal analytical method which is able to solve all the analytical problems in the different fields of application.

Generally, it is not possible to discuss the capacity of an analytical technique without regarding the problem simultaneously. Normally, different analytical methods are necessary to solve the given problems. And by this, the various analytical techniques should be seen as complementary rather than comparative. Always, the problem demands an adequate technique. This includes all the questions about the element, the matrix, the quantity of sample material available for the analysis, the application in practice (routine analysis of research), time consumption (especially important for diagnosis and/or therapy control), price of the analysis, etc. Only combinations of different analytical techniques can help in solving the various problems in trace element analytical chemistry in the biomedical and environmental fields. No serious analyst should try to solve every problem by one technique, which happens to be in the laboratory. This would be in most of the cases a violation of the method and should be avoided for internationally better and comparable results in trace element research and practical application.

In the bio-medical field, the main analytical interest is focused on the well known essential trace elements Co, Cr, Cu, F, Fe, J, Mn, Mo, Ni, Se, Si, Sn, V and Zn in all kinds of tissues and body fluids for clearing up the role and the function of trace elements in the living organism. For some few cases trace element status is used for diagnosis and therapy control of diseases recognized as a malfunction of trace element metabolism. This field of application has a continuously increasing importance due to the increase in the knowledge in this field, for which the development of adequate analytical techniques is the most important factor.

In the environmental field, specific attention is needed for the well known heavy metals, e.g. Pb, Cd, Hg, As, Tl. Another major interest is the interactions of these elements with the essential trace elements in the organism. Therefore not only the pure pollution view is in the foreground, but also the biochemistry of these elements which may have a toxic action in low concentration ranges. But the main point in the environmental field is the control of the environmental load of different substances. In this connection a lot of different matrices – soil, sediments, water, air, aquatic and terrestrial plants, animals and also human tissues and body fluids – are of interest.

In both the application fields, "speciation" analysis is becoming more and more important and demands a combination of inorganic and organic analytical techniques (Steinbrech and König, 1983; Uden and Barnes, 1981).

METHODOLOGY

Modern atomic spectrometry methods can be considered as the most sensitive and specific analytical techniques which are available today for this wide field of application (Schramel et al., 1982). The most commonly used ones are atomic absorption and atomic emission spectrometry. The last mentioned technique is enjoying a renaissance due to the development of the different plasma excitation units, especially ICP, DCP and the different micro-wave induced plasmas (MIP) (Boumans 1978, 1979; Keirs and Vickers, 1977; Skogerboe and Coleman, 1976a, 1976b).

For all the techniques of optical atomic spectrometry, the samples (solutions and/or solid samples) must be converted into an atomic vapour. The sensitivity is strongly dependent on the yield of this process, as are the chemical and physical interferences, i.e. the specificity of the method in general. For the first approach, the atomization of the sample is proportional and the occurrence of chemical and/or physical interferences is inversely proportional to the excitation temperature. Therefore the temperature available in the atomization stage should be as high as possible. The classical excitation sources used in atomic spectrometry like flame, graphite furnace, arc and spark are well known. The temperature available, especially in a flame or in the graphite furnace, is around 3000°C. Due to the Boltzmann-distribution

$$\frac{N_j}{N_0} = \frac{G_j}{G_0} \cdot \exp\left(-\frac{E_j-E_0}{K \cdot T}\right)$$

the main part of the atoms is present in the ground state (G_0). This restriction to low temperatures causes a lot of well known analytical difficulties like chemical interferences due to matrix interferences and molecular absorption or emission. The main reason is an incomplete atomization which is additionally dependent on the chemical environment, i.e. in a graphite furnace or flame. In case of plasma excitation – ICP and DCP sources – the sufficiently nebulized sample will be introduced to the hot zones of a high temperature ionized inert gas (mostly argon) in which the necessary energy is supplied by electrical

Cathode

Excitation Region

Anode

Anode

HF

Sample Introduction

Direct Current
Plasma

DCP

Inductively Coupled
Plasma

ICP
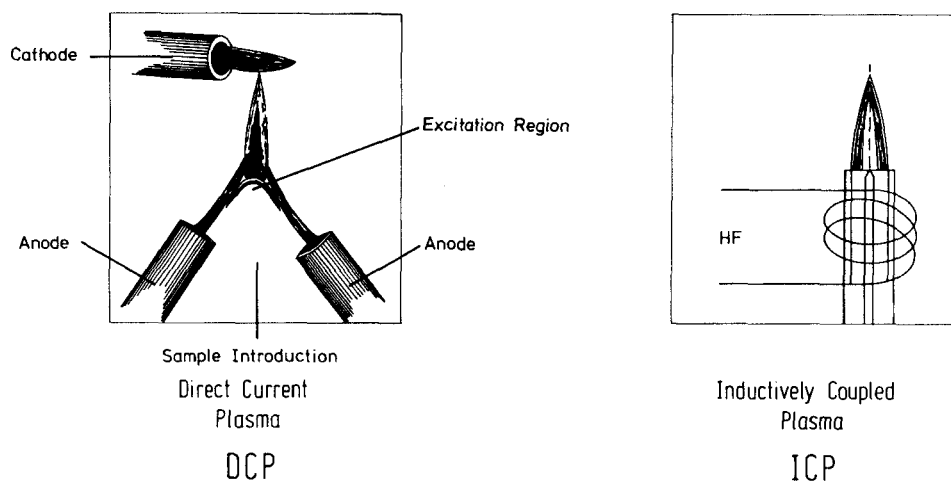
Fig. 1. Schematic drawing of DCP- and ICP-excitation sources.

means (inductively by a high frequency generator or by a relatively high dc-current be-
tween suitable electrodes) as shown in Fig. 1.

In case of ICP, the aerosol passes a tunnel through the plasma. The temperature
available for drying, atomization and excitation will be about 8000 K. In case of the
DCP-source the aerosol hits the surface of the plasma in the center of the three branches.
Due to this, the temperature available is lower than in the ICP and will be about 6000 K.
The yield of atomization is in both the cases higher than in the classical sources due to
the higher temperature, as mentioned before. The relatively high temperature and long
residence times experienced by the sample species and the inert environment provided
by the plasma support gas lead to the expectation that, in comparison to combustion
flames, solute vaporization interferences should be vastly reduced or nonexistent. The
degrees of atomization, as mentioned above, should be more complete, if not approach-
ing 100%, for all metals and metalloids. Because the free atoms are released in a noble
gas environment, depopulation processes such as metal monoxide formation should also
be minimized. These factors combined with the high temperatures should overcome most
matrix interference effects found in flames, arc and spark discharges. The degree to which
these effects may occur depends on some experimental variables, such as height of
observation, carrier gas flow rates and power input to the plasma (Schramel and Xu,
1982, 1984). But the high temperature is also responsible for the very complex nature of
emission line spectra which contain not only the lines from the atoms but also those of
ions (Winge et al., 1979, 1984). This can be an advantage for the analysis. In case of
physical interferences (line coincidences) (Boumans, 1980; Parsons, 1980) at a definite
wavelength of one element, sometimes one can find another sensitive line sufficient for an
interference-free analysis. But due to the complex nature of the spectra the most impor-

tant demand is for an efficient optical spectrometer with a high optical resolution power (Boumans, 1986). Otherwise one has to contend with a lot of physical interferences, especially the mentioned line coincidences which may lead to a lot of systematic errors. But, generally speaking, we have to accept the emission spectra and thus the spectral interferences, which always mean the presence of constituents other than the analyte in the excitation source. Two types of interferences can be observed: a) differences between the calibration functions for the analysis and reference samples and b) changes of the limits of detection and the limits of determination, owing to multiplicative (sensitivity changes) and additive interferences (spectral interferences). Spectral interferences can be attributed to continua, stray light, line wings and lines or bands. Continua and stray light cause a background enhancement and are easy to control by an adequate background correction mode. The interferences caused by lines or band components produce greater problems, because the magnitude of the interfering signals can be determined only indirectly and this determination still forms a fundamental problem in emission spectroscopy. It will be discussed in the section on instrumental parameters. In general one has to test the reliability of the results in a definite matrix on at least a second emission-line or by using an adequate standard reference material. An example of an excellent resolution is given in Fig. 2. It shows the Fe-Quadruplet around 310 nm taken by the JY 38Plus Spectrometer (Instruments S.A.) (Schramel, unpublished, a).

PARAMETERS AND PRACTICAL EXPERIENCE

On the basis of our long experience with two sequential (JY 38 and JY 38 Plus, Instruments S.A.) and one simultaneous ICP-spectrometer (JY 48, Instruments S.A.) as
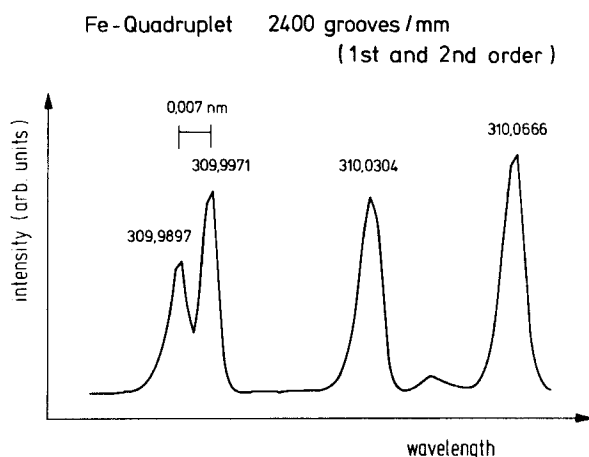
Fig. 2. Resolution of the Fe-Quadruplet taken by the JY38Plus - ICP-spectrometer (slits 25 μm).

well as one sequential DCP-spectrometer (Spectrospan VI, Beckmann) some significant differences and some important parameters will be discussed in the following.

Beside the differences in the excitation sources (ICP, DCP), there exist also differences in the spectrometer itself. The sequential ICP-units have Czerny-Turner arrangements with 1 m focus-length and gratings with 2400 grooves/mm and 2400 grooves/mm (1st and 2nd order) respectively. The simultaneous one has in addition to a 2400 grooves/mm grating a Rowland-circle 1 m in diameter on which the different slits and photomultipliers are mounted. The spectrometer in the DCP unit is an Echelle-spectrometer which is a combination of a 79 grooves/mm grating with an extreme angle of incidence ($\sim 60°$) and a prism (Lautenschläger, 1985). The prism is necessary due to the very poor spectral range of utilization of this special grating which is only about 1 nm in the UV and 15-20 nm in the IR-range. Therefore the prism is necessary for the extension of the spectral range and allows an additional resolution into optical orders (25.-120. order). In this way one obtains a three dimensional splitting of the incident light which overcomes the disadvantage of the poor spectral range. This combination allows the measurement in the range between 150-900 nm.

The spectral dispersion of various gratings is shown in Figs. 3-6 for the example of Zn in presence of Mg ($\lambda = 202, 548$ nm).
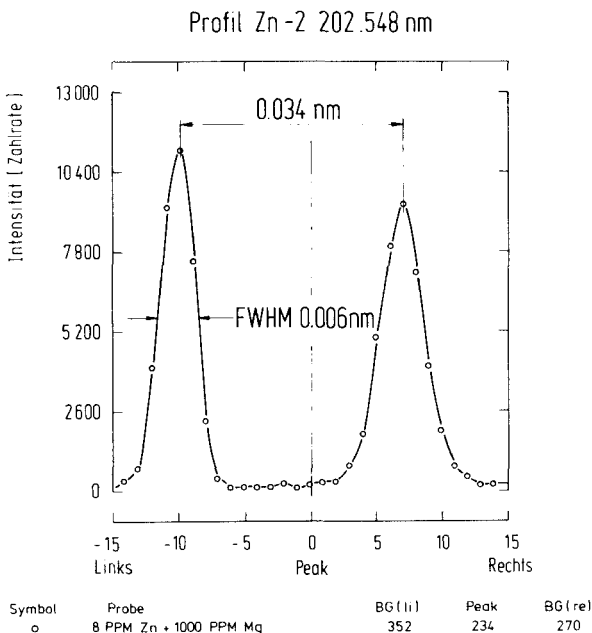
Fig. 3. Spectral resolution power for the Echelle-spectrometer.

P. Schramel

Zn / Mg - ratio : 1 / 500
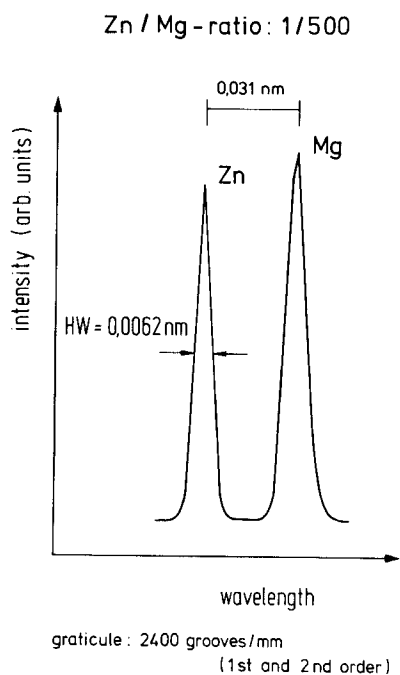
Fig. 4. Spectral resolution power for the 2400 grooves/mm (1st and 2nd order) graticule in the JY38Plus spectrometer.
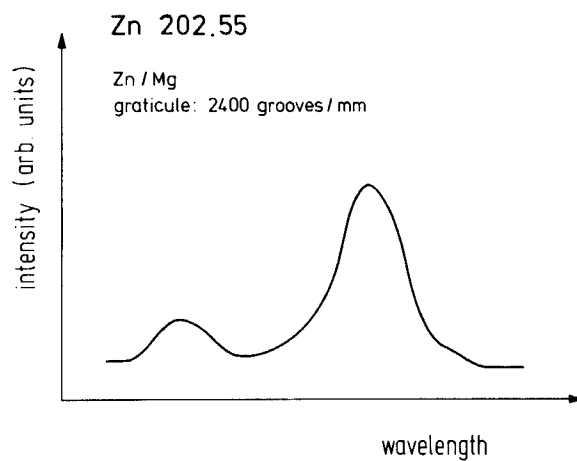
Fig. 5. Spectral resolution power for the "normal" 2400 grooves/mm graticule in the JY38-spectrometer.

Zn/Mg - ratio: 1/500
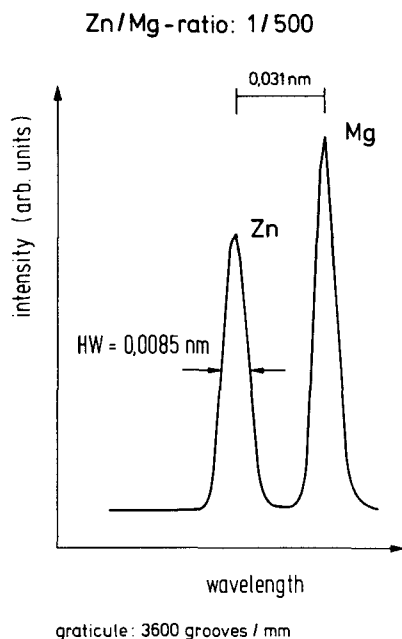
Fig. 6. Spectral resolution power for a "normal" 3600 grooves/mm graticule (other version of the JY38 Plus).

This is only one example of the necessity for a sufficient optical resolution. It would be also very important for accurate background correction as will be described later or for avoiding systematic errors in practical analysis caused by possible line interferences.

Generally, one can obtain similar limits of detection with both the excitation units. In some cases, e.g. for the determination of Al, Li, Si, Pb and Tl, the DCP is more sensitive. The detection limits for Al and Li are about ten times lower in case of DCP compared with ICP. This fact is very useful for the determination of Li in the physiological range in body fluids like blood-serum and urine (Fig. 7) or e.g. for the determination of Al in serum or urine (Fig. 8) or dialysis patients. It is well known that the physiological range of Al in blood-serum is below 5 μg/L and probably below 1 μg/L, so it can not be detected by this method. But in the case of dialysis patients the concentrations in serum increase in some cases (encephalopathy) up to some hundred μg/L, a range which is easily detectable.

This increase of sensitivity in case of DCP is not readily interpretable. Probably, it is a combination of different effects like the lower excess in electrons in DCP, which can be an explanation especially for the alkali-elements, or the lower yield in radiation in the higher wavelength range of a normal holographic grating used in a standard ICP-spectrometer. In case of DCP, higher salt concentrations in the solution lead in some cases to a higher sensitivity (Cd, Pb, Tl, Zn). For examples, the detection limit for Tl improves from 50 μg/L to about 1 μg/L in presence of high salt concentrations. It is well known that with ICP-
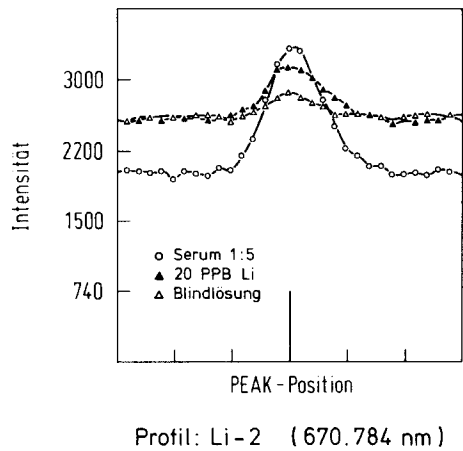
Fig. 7. Example for Li-spectra in blood-serum obtained by DCP-spectrometer.
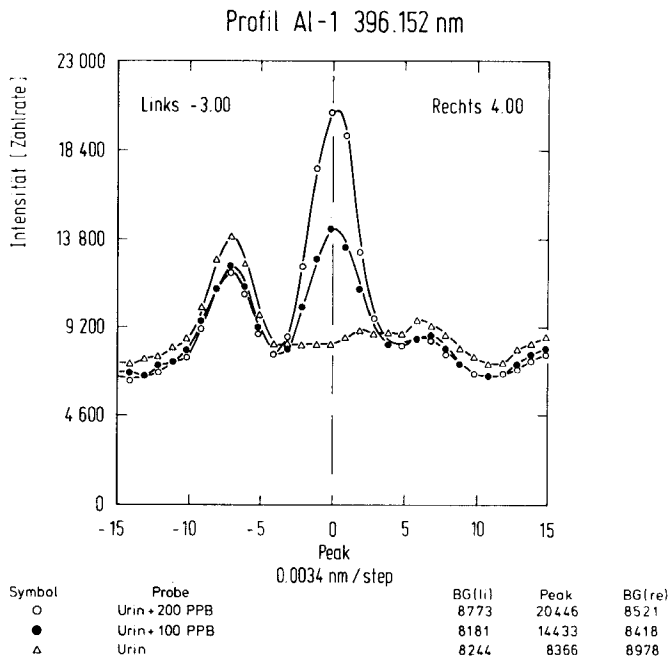
Fig. 8. Example for Al-spectra in urine samples obtained by DCP-spectrometer (standard addition).

TABLE 1

COMPARISON OF RESULTS ON IDENTICAL SAMPLE SOLUTIONS (1645 = NBS river sediment; 1632 A = NBS coal; SL2 = BCR sewage sludge; S06 = BCR soil).

Sample/element		Fe (%)	Mn (μg/g)	Zn	Cu	Ni	Cd	V
1645	ICP	11.5	740	1715	105	85	8.3	45
	DCP	11.4	760	1670	115	50	10.3	21
cert. value		11.3	785	1720	109	45.8	10.2	23.5
1632A	ICP	1.09	25	32	18	35		65
	DCP	1.08	27	27	19	18.5		44
cert. value		1.11	28	28	16.5	19.4		44
SL2	ICP	0.9	270	3200	430	65	15	41
	DCP	0.8	270	2800	400	43	17.5	15
cert. value		0.8	241	2843	429	41.4	18	16.6
S06	ICP	2.5	995	1500	260	140	36	155
	DCP	1.9	990	1220	220	105	35	88
cert. value		2.0	999	1272	236	99.5	31.1	

excitation just the opposite occurs. But the reasons for these effects are not well investigated up to now and therefore difficult to describe and interpret (Schramel, 1986).

Table 1 shows some results of comparative determinations with both the techniques on identical sample solutions of different standard reference materials (SRMs) with relatively difficult matrices (1645 = NBS river sediment; 1632A = NBS cool; SL2 = BCR sludge; S06 = BCR soil). The determinations have been done on the simultaneous ICP and compared with those of the DCP-spectrometer. It is easy to see that some differences exist for "problem-elements" (with regard to the spectral resolution power of the ICP) like Cd, Ni and V. In those cases interelement correction is necessary due to line interferences. As will be described later the correction factor is determined by measuring the share of the disturbing element to the counting rate of the element of interest. In all cases the software corrections are based on a linear course of the disturbance, but unfortunately this assumption does not work in practice. Therefore the correction factor is always valid only for one definite concentration ratio of the disturbed to the disturbing element. Therefore systematic errors can be observed for different ratios. In these cases, the advantage of higher spectral resolution power can be easily seen due to the reduction of the need for interelement corrections. Of course, this effect is independent of the excitation source. One of the most important disadvantages of DCP-excitation for practical measurements is the effect of line inversion. That means high element concentrations in the cool zone of the plasma absorb the radiation of the hot zones of the plasma and

TABLE 2

SINGLE CELL PROTEIN (BCR/EG). DETERMINATION BY ICP- and DCP-AES WITH AND WITHOUT Li-PUFFER.

Sample/element	Ca	K	Mg	P
ICP	12240	—	2580	24530
DCP	8840	1297	2565	24970
DCP (Li)	13260	929	2565	24270

therefore the detected intensity in the spectrometer is lower than the corresponding real analyte concentration. This effect can be strongly observed in K-determinations in blood-serum or urine. Therefore one has to use Li (10 g/L) or Cs (1 g/L) as an ionization puffer for getting true results.

Table 2 shows an example of it in the determination of the main elements in a single cell protein. This effect can not be observed for the ICP in practice. Generally one can say that, probably due to the lower excitation temperature available for the sample in case of DCP compared to ICP, the chemical matrix interferences become more serious. Therefore it is necessary to test the analysis with adequate SRM's to avoid systematic errors and emphasize the demand for an excitation temperature as high as possible as described in the beginning.

The following discussion of some important operating parameters is mainly restricted to ICP-spectrometers, although some of these points are generally accepted:

1. RF-generator

Nearly all manufacturers of ICP-instruments offer RF-generators in the power range between 1-5 kW. The analytical problems must be examined carefully for the right choice. A power of 1.5 kW would be sufficient for aqueous solutions and inorganic solvents. But in case of organic solvents the RF-power must be at least 2.5 kW. Generally, one can observe a faster increase of the spectral background with the RF-power in comparison to the line signal. Therefore the signal/background ratio, which is one parameter describing the detection limit, decreases with increasing power. In practice an RF-power of about 1 kW delivers the best conditions with regard to detection limits, stability etc. in the application field under discussion (aqueous solutions). Another point is the frequency of the RF-generator. In the past, the frequency commonly used was 27.12 MHz; now other types of generators have become available and the trend moves to higher frequencies, about 40 MHz. In our own experience, one of the most significant advantage of 40 MHz in comparison to 27 MHz is a significantly lower background, especially in the wavelength range between 170-300 nm (Fig. 9).
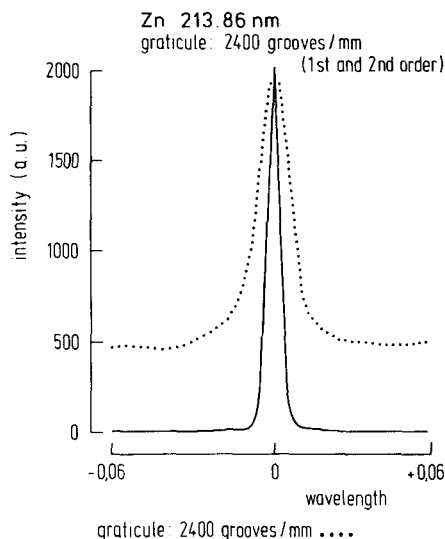
Fig. 9. Comparison of the background of a 27 MHz ($\cdots$) and a 40 MHz) (———)
RF-generator.

Therefore the signal/background ratio and the detection limits improve significantly,
generally by a factor of 2 or 3.

2. Simultaneous or Sequential Spectrometer

Answering this question one must take into account the problems to be solved by the
instrument. For routine analysis, working always with similar samples (matrix) and deter-
mining the same elements, one would favour a simultaneous spectrometer attending to
two important points: a) for the matrix under consideration, adequate wavelengths for the
elements which should be determined must be chosen for an interference-free detection
and b) the possibility for a sufficient background measurement and correction must be
installed. Otherwise it would always be a source for strong systematic errors. The back-
ground varies dependent on the composition of the sample or on the sample solvent etc.
(Fig. 10). When emission spectroscopy is mainly used for research work or solving rapidly
changing problems concerning both the elements and the matrix, one would prefer a
sequential instrument or at best a combination of both types. Nearly all the elements have
different emission wavelengths with comparable intensities. Therefore a sequential instru-
ment offers the possibility of checking interferences from disturbing elements, the check
of adequate position of background correction and thus the choice of the wavelength for
lest biased measurements. The main disadvantages of a sequential device are the higher
consumption of sample-solution and the increasing time for one analysis, dependent on
the number of elements which would be determined (in practice : e.g. for 17 elements : 5
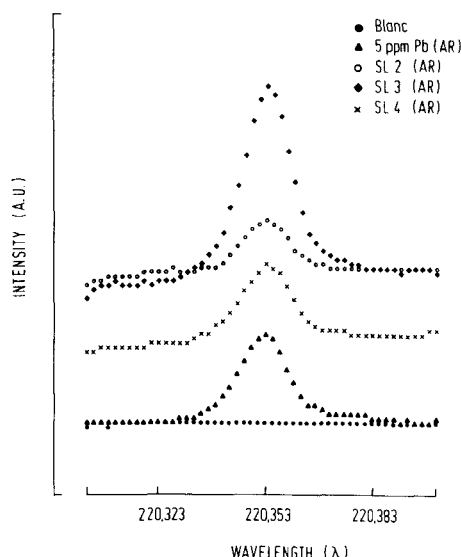
Fig. 10. Variations of the background in dependence on the matrix composition (on the example of different sludge-samples).

min for simultaneous, 9 min for sequential determination). The promises of the manufacturers, 20 elements or more per min, can not be realized in real samples and with an acceptable standard deviation.

3. Aerosol-generation

For the continuous operation of an ICP, two different types of nebulizers are available: the pneumatic nebulizer with all the more or less important different constructions and the ultrasonic nebulizer.

Both types have been investigated in detail (Olson et al., 1977; Greenfield et al., 1976, 1977; Winefordner and Latz, 1961; Schramel and Ovcar-Pavlu, 1979). In principle, using a pneumatic nebulizer, one should apply a peristaltic pump for the transport of the sample solution. Such a pump reduces the sample consumption significantly in comparison to a free sucking system (appr. 1 mL/min as against appr. 5 mL/min) and reduces the influence of viscosity and density of the sample solution to the droplet-size which will be produced by the system due to the amount of solution forced through the capillary. Therefore an important influence on the sensitivity (expressed as the slope of the calibration curve) can be nearly eliminated.

The "Poisseuille-Law" is valid for the amount of liquid which can be transported through a capillary.

$$Q = \frac{\pi \cdot R^4 \cdot P}{8\eta L}$$

in which Q is the amount of liquid, R the radius of the capillary, P the difference in pressure, η the viscosity, and L the length of the capillary.

The amount of liquid and the droplet size created by the nebulizer system are the most important factors influencing the sensitivity. The droplet size itself is also dependent on density, viscosity and surface tension of the solution. An empirically derived formula was given by Nukiyama and Tanasawa (1938-1940).

$$d_0 = \frac{585}{v}\left(\frac{\sigma}{\rho}\right)^{1/2} + 597\left[\frac{n}{(\sigma \cdot \rho)^{1/2}}\right] 0.45\left(1000\frac{Q_{liq}}{Q_{gas}}\right)$$

in which d_0 is the mean droplet diameter, v the velocity of the gas stream, σ the surface tension, ρ the density of the liquid, and n the viscosity of the liquid.

Fig. 11 shows the effect on the example of an HCl-solution.

The main advantage of an ultrasonic nebulizer is the significantly smaller droplet size and the subsequent improvement in sensitivity for nearly all elements by a factor between 5 and 50 (Schramel et al., 1982). The droplet size created by an ultrasonic nebulizer is given by the following formula by Robin (1977):
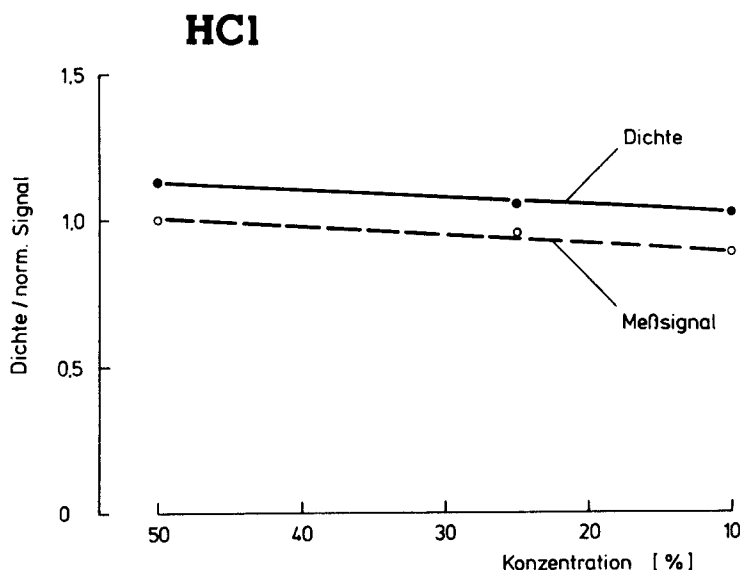
Fig. 11. Dependence of the signal on the density of the solution (ICP, pneumatic nebulizer).
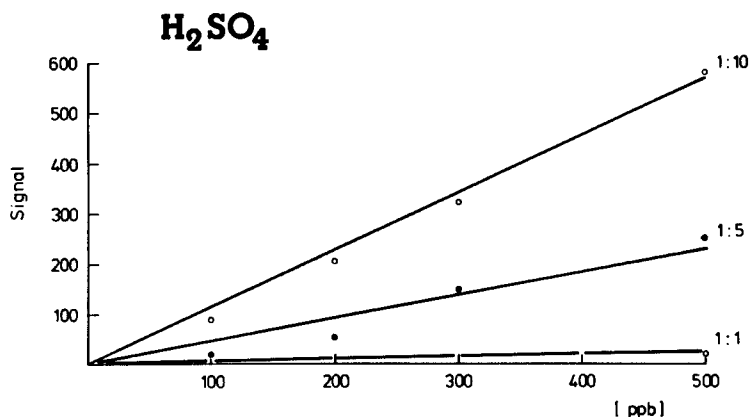
Fig. 12. Dependence of the slope of the calibration curve on the acid concentration (ICP; ultrasonic nebulizer).

$$d_0 = 0.34 \frac{(8\sigma)^{1/3}}{\rho \cdot f^2}$$

with ρ the surface tension, σ the density, and f the frequency of the ultrasonic nebulizer.

The disadvantages are the higher memory-effect, whereby it takes a longer time for washing out highly different concentrations in the sample solutions between two different samples, and a stronger dependence on the sample solution, e.g. signal-differences by introducing solutions with different acid concentrations (Fig. 12) (Schramel and Ovcar-Pavlu, 1979).

The application of a peristaltic pump in the sample introduction system in case of the ultrasonic nebulizes is necessary in each case.

Other techniques like hydride-generation, flow-injection techniques or solid sample introduction are highly specialized and up to now not applicable to routine analysis.

4. Plasma-gases

For normal operation of an ICP source, at least three different gas flows are necessary: cooling-, plasma- and aerosol carrier-gas. For special constructions of the "torch", other gas flows, e.g. a shielding gas, are used. Normally, argon is applied. For the improvement of the long- and short-time stability, special care must be taken with the gas regulating system, because all variations in the different gas flows influence the plasma and thus the emission conditions (e.g. observation height, RF-power, background etc.). Most sensitive in this connection is the carrier-gas flow. The normal needle valves and suspension-body flowmeter are not sufficient for an adequate operation of an ICP- or DCP-source.

The application of electronic mass flowmeters and controllers is best. They are independent of pressure and temperature in a wide range and guarantee a perfect stability of

all gas-flows, leading to a strong improvement of the stability of the plasma source (Schramel, 1985).

Admixing a small amount of hydrogen to the argon, especially in case of carrier-gas (about 3% of the total amount), improves the detection limits in the wavelength region between 200-400 nm by a factor of 2-10 applying ion-lines (Fig. 13) (Schramel and Xu, 1984; Schramel et al., 1981).

Four important statements have been derived from these measurements:

a) The net peak area can be increased significantly, especially in the wavelength region of 200 to 300 nm, by small additions of hydrogen to the different gas-flows of an argon ICP.

b) The stability of the plasma, expressed as the standard deviation of the background, can be improved under the same conditions over the entire measured wave length region.

c) Owing to a) and b) the detection limit of all the elements determined as shown in Fig. 13 can be improved.

d) The effect is dependent on the wavelength and on the excitation state of the atom. From the measurements (Fig. 13), the relationship can be assumed to be logarithmic in both cases, which is more significant for ion lines than for atom lines. A typical "saturation" effect was obtained in both states over 400 nm.

In conclusion, small amounts of hydrogen (appr. 3% of the amount of argon) added to the different gas flows used in an ICP source significantly affect the behaviour of an ICP.

The first assumption, that this effect is produced by a higher energy transport from the plasma to the aerosol due to the higher thermal conductivity of hydrogen compared to argon, could not be confirmed by admixing helium to argon. The thermal conductivity of helium is comparable to that of hydrogen but it would be present in atomic form in the plasma and therefore more thermal energy should be available for the atomization and
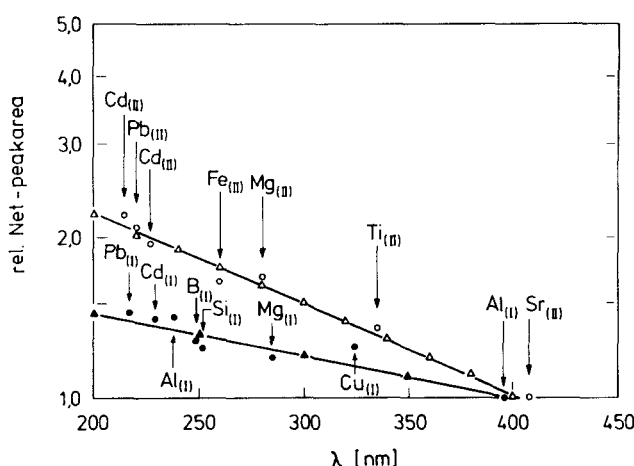
Fig. 13. Effect of H_2-admixture to Ar-carrier gas in case of ICP.

excitation of the aerosol and atoms. So, the effect may be produced by a geometric variation of the plasma which leads to a higher density of the atomic vapour.

A completely different behaviour can be observed in case of DCP. For it, one can obtain a strong dependence on the excitation energy needed independent on the excitation state (atom or ion) and therefore it may be in this case an effect of an higher energy transport from the plasma to the aerosol. Unfortunately hydrogen admixed to the argon can not be used for a longer time in the DCP-unit due to an increased consumption of the graphite electrodes. A small amount of helium (appr. 10%) to the argon burning gas improves the stability of a DCP source and by this an improvement of the detection limits by appr. a factor 2-3 can be reached.

Most important for the short- and long-time stability of a DCP source is the use of electronic mass flow controllers and meters, resulting in a stable observation zone which is much more smaller than in an ICP.

5. Optimization of operating parameters

In principle, nearly each element and each sample solution has its own best operating conditions, concerning especially RF-power, observation height (dependent on the excitation conditions) and nebulization parameters (dependent on the density, viscosity of the sample solution). For practical reasons (simultaneous or sequential determination of more than one element in a given sample solution), it is always necessary to work under compromise conditions. Experience shows that optimization of the operating parameter can be done using Fe (λ = 259.94 nm) or Ca (λ = 393.37 nm) (Schramel and Xu, 1984). The commonly used parameters describing the optimization stage are Signal/Background-Ratio (should be as high as possible), the Background-Equivalent-Concentration (BEC; should be as low as possible) and the variation of the background (should be as low as possible). All three parameters strongly influence the detection limit. Beside these, one has to look for spectral interferences (line overlapping) in each individual sample. They would strongly influence the limit of determination (Schramel and Xu, 1984).

6. Evaluation methods of the signals

In most of the commercially available instruments, 5 different calculating routines for the qualitative detection of the element-signals are controlled by the software (Fig. 14):

a) Determination of the peak-height at a fixed wave-length position

The measurement is done at the maximum of the peak only (N_{eff}) which is found by a previous peak-search run and stored in the wavelength table as the actual peak position. Background correction is also done at one or at both previous fixed positions on the left (N_1) and right (N_2) side of the peak. According to this 3- or 2-point measurement, the net-signal will result from
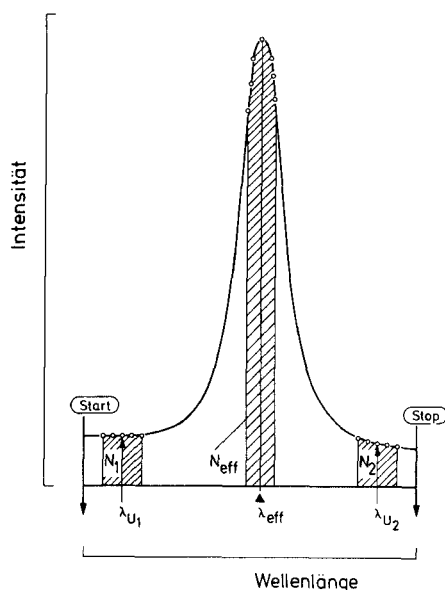
Fig. 14. Evaluation methods for emission-lines.

$$N_{net} = N_{eff} - \frac{N_1 + N_2}{2} \quad (\text{or } N_{net} = N_{eff} - N_1(N_2))$$

This procedure is very fast but has an important disadvantage: small variations in the actual peak position due to temperature variations in the monochromator box or due to electronically derived counting errors in the step motor of the monochromator drive are directly reflected in strong variations of N_{eff}. This leads to a bad standard deviation and a bad reproducibility of the measurements. Therefore this evaluation method should be used only for semiquantitative measurements.

b) Determination of the peak-height at variable wave-length position

Additionally to the measurement as described above, a free selected number (mostly 3-11) of channels is measured (scanned) and the highest value in this window is taken as N_{eff}. This evaluation procedure can be well applied to concentrations clear above the detection limit (by at least a factor of 100), otherwise the counting statistic will influence the "highest value" and also lead to a higher relative standard deviation (3-5%). On the other hand, the advantage is a relatively fast measurement and therefore a low sample consumption. Especially in case of biological and medical samples, when only small

amounts of material are available for the analysis, this procedure is the only possible compromise.

c) Determination of the peak-area

A free selected region around the peak position (as stored in the wavelength table) will be scanned (about 50 channels, dependent on the scan-width) and the peak area will be calculated as the sum of the intensities of a free selected number of steps around the peak maximum found during the scan. The background will be found either by the same procedure automatically looking for the minimal on the left and on the right side of the peak or by measuring at fixed positions. This evaluation procedure leads to a better precision and reproducibility of the measurements (< 1%), especially near the detection limit, but takes more time for the measurement and therefore leads to a higher sample consumption (factor 3-4 more). This method is dangerous to use when, e.g. one disturbing peak from another element occurs in the scanning area, which is higher than the peak of the element which should be measured. In this case, a good measurement is then dependent on the capacity of the software.

d) Gauss-curve fitting

This evaluation procedure is more of academic interest. In principle, it is the same scanning procedure as described in c) but starting from the measured intensities around the maximum a Gauss-curve is calculated for the peak from at least 3 points from which the peak maximum and the peak area is derived. This calculation works sufficiently only in case of very clear peaks (clear above the detection limit), but takes additional time.

In practice – depending on the problem and the amount of sample available for the analysis – the methods described in b) or c) should be applied.

e) Interelement correction

Most of the software commercially available for ICP-spectrometers has the possibility for interelement corrections. In some of the given problems, the possibility to measure the element of interest at an undisturbed wavelength does not exist, which means this element will be influenced by another by peak overlapping. In this case, it is necessary to determine the influence of the disturbing element on the disturbed element by calculating a correction factor. This is done by taking different calibration curves of the element of interest, adding different concentrations of the disturbing element (Fig. 15).

Obviously, the concentration of the disturbing element must be measured always at another wavelength, therefore the measuring time will be extended. The main disadvantage of this correction comes from the non-linearity of the correction factor as it is dependent on the concentration of the disturbing element. All the commercially available software systems in the ICP proceed on the assumption of a linear course of the correc-
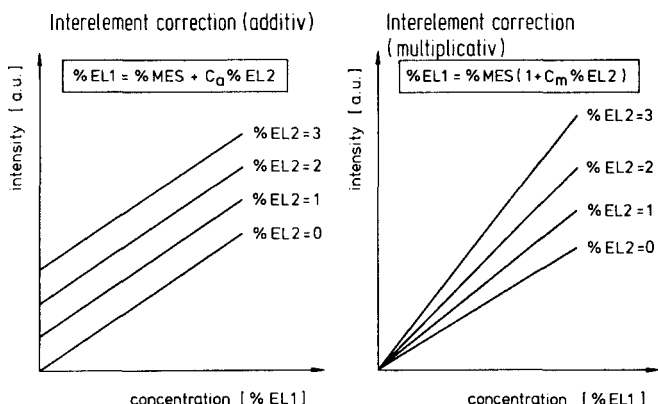
Fig. 15. Example for the determination of interelement correction factors.

tion. Therefore systematic errors result at concentration ratios far from the ratio for which the factor was determined.

For this reason, the interelement correction should be avoided by using an undisturbed emission wavelength or by using a spectrometer with a high optical resolution power.

f) Internal standard

A very useful method for overcoming various influences to the measured emission signal such as stability problems due to the different gas-flows, changes in the RF-power, or nebulizer effects (density and/or viscosity of the sample solution) is the application of the internal standard technique. A known amount of an element which is usually not present (or at a very low concentration level which does not influence significantly the added amount) in the unknown samples is added to the calibration solutions and to the samples. This element should be determined simultaneously to the element(s) to be determined. Its signal is then used to standardize each other signal. The best way is to measure the internal standard with a second independent monochromator. When the internal standard is measured sequentially in the measuring cycle of the elements to be determined, only physical influences due to different density or viscosity of the samples can be corrected by this method, but not short- and long term instabilities.

In this connection, the application of adequate standard reference materials (SRM's) for testing the accuracy must be pointed out. Regarding such difficulties as described above, it is obvious that SRM's taken for such an examination must be very similar to the samples to be analyzed in the matrix composition and in the concentrations of the elements under investigation. This demand requires the availability of many different SRM's for all kind of sample materials and all analysts should strongly support the serious

activities of the different national or international institutions such as the NIST (National Institute of Standards and Technology, U.S.A.), BCR (Community Bureau of Reference of the European Communities) or others in producing and certifying such materials.

The use of certified SRM's is one of the important steps in quality control procedures (Koh et al., 1980; Michel et al., 1983). Another possibility is the use of an SRM as multielement standard for calibrating an analytical device, especially here a plasma emission spectrometer (Schramel and Xu, 1983). The use of "synthetic" standards, where known amounts of elements are prepared is the predominant method in the laboratories (Chaplin and Dicon, 1974; Scott and Strasheim, 1975; Mc Quaker et al., 1979). But this technique has a number of limitations including the preparation, dilution and mixing of a large number of different primary standard solutions with subsequent errors such as contaminations caused by the impurities in the element compounds added as well as the problem of stability during storage over a long time. Sometimes, it is also necessary to match approximately the matrix composition of the samples to obtain satisfactory accuracy of determination. These difficulties can be overcome by using a well certified SRM for calibrating the spectrometer. The main demand in this connection is the use of an SRM sufficient for this purpose. It must be very similar in the matrix composition and in the elemental concentration ranges to the unknown samples. Knowing the wide variations in the concentrations of mineral and trace elements in biological and environmental samples it is for example not possible to compare one plant with another and therefore also in this case it is not really possible to work without interelement corrections. They must be introduced due to the normally wide range of ratios of different elements which influence one another (Schramel and Xu, 1983).

SPECIAL REMARKS ON THE DCP-PLASMA SOURCE

The mechanical configuration that contains or supports the plasma is quite different to ICP. The DCP (Fig. 1) uses a multiple electrode set: two graphite anodes and one tungsten cathode. Each electrode is cooled by flowing argon. The electrode set tends to lock the plasma volume in position, which provides much of the stability of the generated signals. For this reason, improvement of the stability can be reached by using mass-flow-controllers for all gas flows in a DCP-source, similarly important as for an ICP, as mentioned before. In case of ICP, there is a relatively constant power density over its typical operating range due to the large size of the ICP, in which power or volume fluctuations cause very little alterations of the power density. Another picture occurs in the DCP: The device is current not power controlled. Consequently, the power density can fluctuate if the resistance of the material in the electrical gap changes measurably.

High concentrations of easily ionized matrix elements, for example, can alter the ohmic heating zones of the discharge. The resultant swelling of the generated plasma volume results in reduced power density. The consequence is a temperature drop of the discharge. A result of the size of the generated plasma volume is the size of the viewing zone for the spectroanalytical observation. The DCP viewing zone is about 8 mm^2, in case of

ICP, it is tens of square millimeters. Therefore, fluctuations of the viewing zone cause stronger influences in DCP than ICP.

APPLICATIONS OF PLASMA EMISSION SPECTROSCOPY: BIOMEDICAL AND ENVIRONMENTAL

a) Biomedical samples

Trace element research in the biomedical field is mainly focussed on the known essential trace elements Co, Cr, Cu, F, Fe, J, Mn, Mo, Ni, Co, Si, Sr, V and Zn. For investigations on more toxicological aspects or for questions of the interactions with other trace elements in the organism or in general for questions of the environmental load, the known heavy metals such as Cd, Hg, Pb, Tl etc. are of interest. In the field of occupational health, all elements which occur in the workplace can be of interest.

It is not possible to discuss here all special cases and problems. Therefore, the following statements are restricted to some important body-fluids and organs and applying plasma emission· spectroscopy. In general, the "normal" concentrations of the heavy metals in these samples can not be determined by plasma emission spectroscopy due to very low concentrations which are in most cases far below the limits of determination (exception: sometimes liver and kidney for Pb and/or Cd).

Whole Blood has in practice no significance for diagnosis and for therapeutic monitoring using the trace element status. Exceptions are trace elements which have a special bounding to the erythrocytes, e.g. Cd and Pb, but the concentrations present are too low for ICP-AES. Besides, in most cases a mineralization or digestion of the whole blood sample is necessary, because it is not possible to introduce it into a normal pneumatic nebulizer.

Milk (cow and human breast milk) plays an important role in nutrition, especially for infant food. All essential trace and mineral elements are under investigation. The analysis of the mineral elements Ca, K, Mg, Na and P presents no difficulties. Of the essential elements only Cu, Fe, Mn and Zn are detectable. Using a hydride generation system, one can also determine Se. A mineralization step is mostly necessary. An exception would be in applying an ultrasonic nebulizer, but further developments on it are necessary for routine applications (Table 3,4) (Schramel, 1979).

Liquor, amniotic fluid and bile samples are commonly available only in very small amounts (1-5 mL) and in case of a definite medical indication. Therefore, no sufficient reference values about "normal" trace element concentrations are available (Table 5). Besides the risk for contamination during the routinely used sampling procedure is extremely high.

The body fluids mainly used for the investigations of the behaviour of trace elements in the organism and for diagnosis and therapy control are **blood-serum** (or -plasma) and **urine**.

TABLE 4

CONCENTRATION OF TRACE ELEMENTS IN HUMAN BREAST MILK (μg/L) MEASURED BY ICP-AES (EXAMPLE).

Sample/element	Cu	Fe	Zn
Typical values	400	340	5000

TABLE 3

CONCENTRATION OF TRACE ELEMENTS IN DIFFERENT MILK-POWDER SAMPLES (ng/g DRY WEIGHT) MEASURED BY ICP-AES (EXAMPLES).

Sample/element	Cu	Fe	Mn	Zn
1	190	3600	550	550
2	1000	2400	2000	1600
3	2700	16500	2000	5000

In **serum**, the essential trace elements Cu, Fe, Zn and the macro-elements Ca, K, Mg, Na and P can be easily determined by plasma emission spectroscopy (Table 6).

In this connection one must point out the importance of multi-element analysis in serum for diagnostic purposes. The determination of a single trace element gives no evidence about the relative abundance of this element, because many factors influence the "normal" actual trace element status: stress situations, nutrition etc. The trace elements are mostly bound to proteins and therefore it should be standardized to the total protein content (Fig. 16). In practice, the phosphorus concentration in serum is a sufficient

TABLE 5

CONCENTRATION OF MAIN ELEMENTS IN BILE (mg/L) MEASURED BY ICP/DCP-AES (EXAMPLE).

Sample/element	Ca	K	Mg	Na	P
Typical values	80	150	20	3500	120

TABLE 6

REFERENCE VALUES IN HUMAN BLOOD SERUM (mg/L)

Cu	:	1.0-1.3
Fe	:	1.0 (haemolysis!)
Zn	:	0.8-1.1
Ca	:	100-120
Mg	:	19-24
Na	:	3200-3600
K	:	140-180
P	:	120-150

value for this standardization, because more than 85% of phosphorus is bound to pro-teins. Only by performing multi-element analysis, can wrong conclusions about the trace element status be avoided (also in case of haemolysis).

Two other elements which are of interest in the medical field, namely Al and Li, can be determined by DCP-spectroscopy more sensitively (factor 10) than by ICP.

Al is of interest as a toxic metal for uremic patients and must be controlled in the blood serum continuously. The "normal" Al concentration in serum is far below 5 μg/L, probably below 1 μg/L. In case of dialysis patients values up to 1000 μg/L can be observed (Fig. 17). The Figure shows also the necessity of background correction in general, and that strong variations of the background level due to the matrix can occur.

Li is under discussion as being essential for man (Anke et al., 1984). Determinations (Schramel, unpublished, b) have shown a mean value for the concentration of about 50 μg/L with a very sharp distribution. This can be an indication for a homeostatic control mechanism and thus to an essential effect in the organism.

The benefit of urine analysis in clinical chemistry and occupational health medicine for diagnosis and therapy control is indisputable. Plasma emission spectrometry can deliver a lot of possibly important information about trace element concentrations, which can not be obtained simultaneously by any other analytical technique (Schramel et al., 1985).

For significant estimation of such analytical results, it is necessary to take into account only the total excretion per day of trace elements. The concentrations of trace elements in spontaneously taken urine-samples are strongly dependent on the nutrition behaviour of the probands (Fig. 18).

ICP- and or DCP-emission spectrometry allow the determination of the mineral ele-ments Ca, Mg, Na, K, P and the trace elements Li and Zn without any difficulties. Cu and Fe are present in a concentration range of about 10 μg/mL and therefore near the detection limit for most of the instruments (Table 7). The elements Ba, Sr and Ti are of interest from the occupational exposure point of view (Schramel, 1987). Also, the physio-logical range for Ba (mean value: 2.8 μg/L) and for Sr (mean value: 204 μg/L) can be estimated (Fig. 19,20). The "normal" value for Ti is below 1 μg/L and can not be detected (Fig. 21). An external load can be estimated for all three elements.
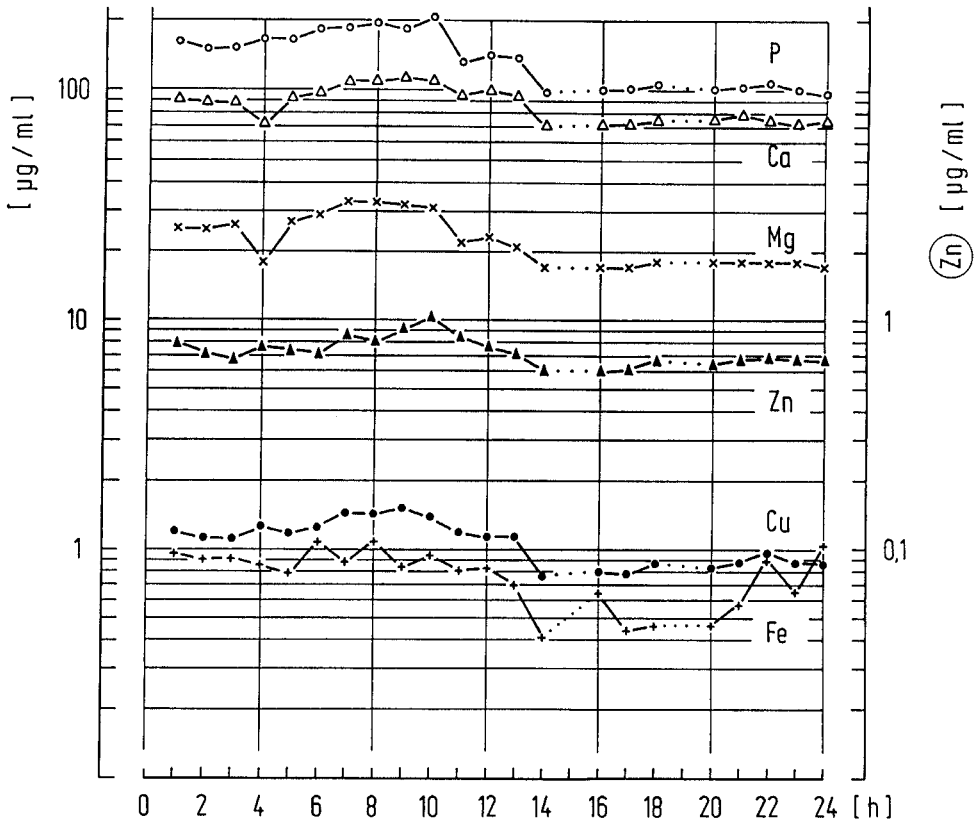
Fig. 16. Variations of trace and mineral element concentrations in human blood-serum during 24 hrs (example).
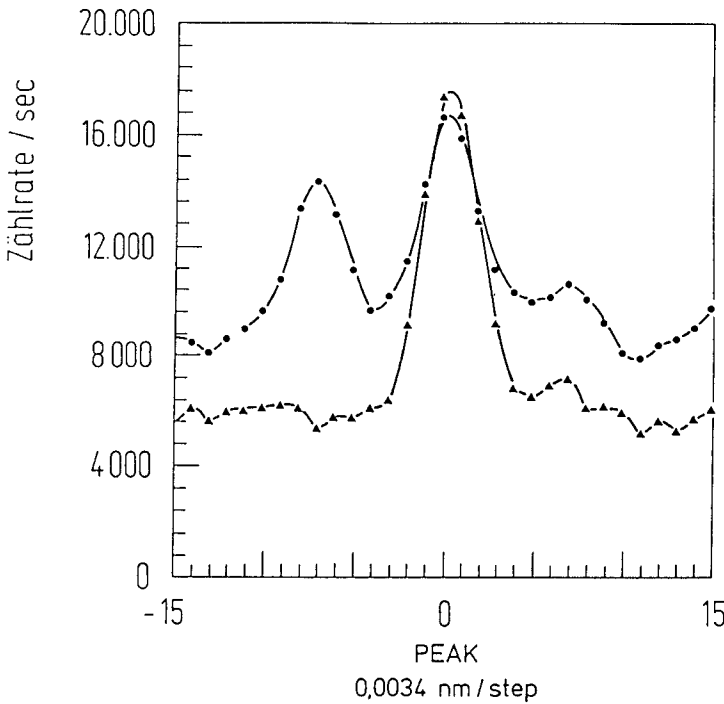
Fig. 17. Al-spectra from blood-serum of a dialysis patient (●) in comparison to 100 mg/L aqueous standard solution (o) (example).
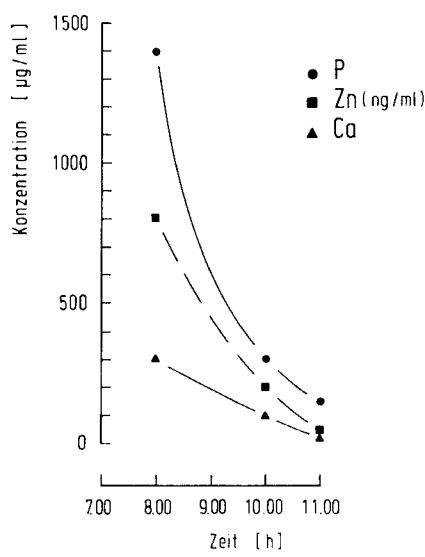
Fig. 18. Dependence on sampling time of the concentrations of different elements in urine.

TABLE 7

CONCENTRATIONS OF MINERAL AND TRACE ELEMENTS IN HUMAN URINE SAMPLES

element	method of determination	number (n)	mean value (μg/L)	SD	mean value (mg/L)	SD	range (μg/L)	range (mg/L)	Boumans (1986) (μg/L, mg/L*)
Al	DCP	50	15	12	—	—	5-30	—	300
Ba	ICP/seq.	25	4.5	4.2	—	—	0.2-12.7	—	10
Ca	ICP/sim.	105	—	—	195	82	—	70-390	280*
Cd	Voltammetry	50	0.8	—	—	—	—	—	20
Co	Voltammetry	50	0.45	—	—	—	—	—	—
Cu	ICP/sim. DCP	105	12	3.4	—	—	2-20	—	350
Fe	ICP/sim. DCP	105	11	14	—	—	2-70	—	250
K	DCP	54	—	—	1982	640	—	660-3270	2300*
Li	DCP	40	58	10	—	—	10-300	—	300
Mg	ICP/sim. DCP	105	—	—	135	79	—	40-426	175*
Na	ICP/seq. DCP	40	—	—	3584	1370	—	1590-7022	2840*
Ni	Voltammetry	50	1.5	—	—	—	—	—	100
P	ICP/sim. DCP	105	—	—	525	288	—	220-1070	360*
Pb	Voltammetry	50	21.5	—	—	—	—	—	50
Sr	ICP/seq.	25	240	181	—	—	27-798	—	400

Ti	ICP/seq.	25	1	—	—	—	—	—	100
Zn	ICP/sim.	105	450	240	—	—	180-850	—	450
Creatinin according to Jaffé		50	—	—	1.3[+]	0.45	—	0.44-2.41[+]	—

[+] g/L

In case of tissue analysis, the number of elements which can be determined by plasma emission spectrometry is again dependent on the kind of tissue, due to the very different concentrations of the elements in the organs or tissues. In most cases, it is – also here – only possible to determine the trace elements Cu, Fe, Mn and Zn, in addition to the mentioned mineral elements. Table 8 shows examples for these at the hand of typical SRM's produced by the BCR (Wagstaffe et al., 1986).

In spite of the fact that the total number of essential trace elements detectable by ICP- and or DCP-emission spectrometry is relatively small, this analytical technique has a considerable capacity in this field of application, above all due to the possibility of multi-element determinations.

b) Environmental analysis

In the field of the determination of environmental samples, one has a wide spectrum of different sample-matrices: air and dust, water (drinking water, surface water, waste water etc.), soils, sediments, sludges, plants, animals and their organs. Due to the mostly higher concentrations of relevant elements – dependent of course on the sample material – one can be able to determine more relevant trace and mineral elements by plasma emission spectrometry. Again, it is impossible to discuss here all the possibilities. Therefore some selected examples from the practice of environmental analysis will be given:
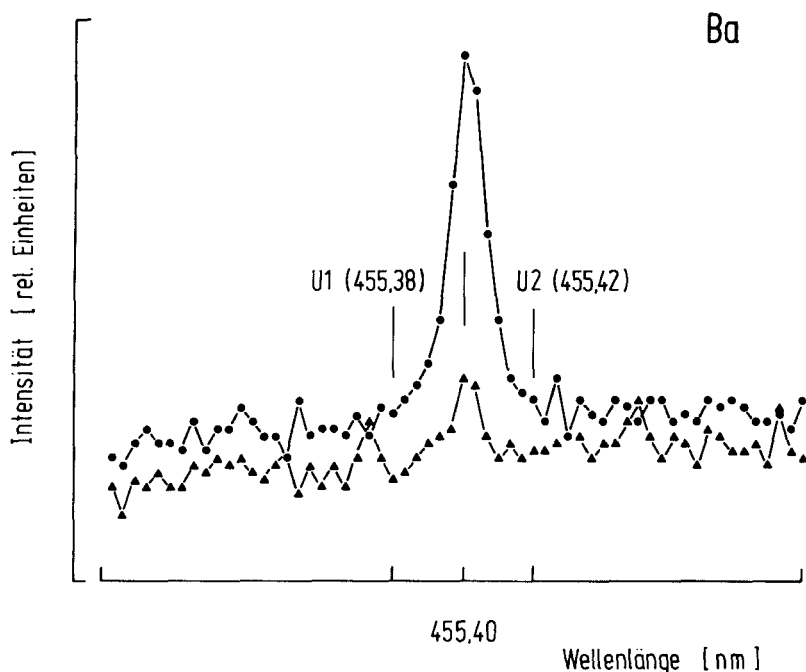
Fig. 19. Ba-spectra in human urine (▲) and standard addition of 10 g/L Ba (●).
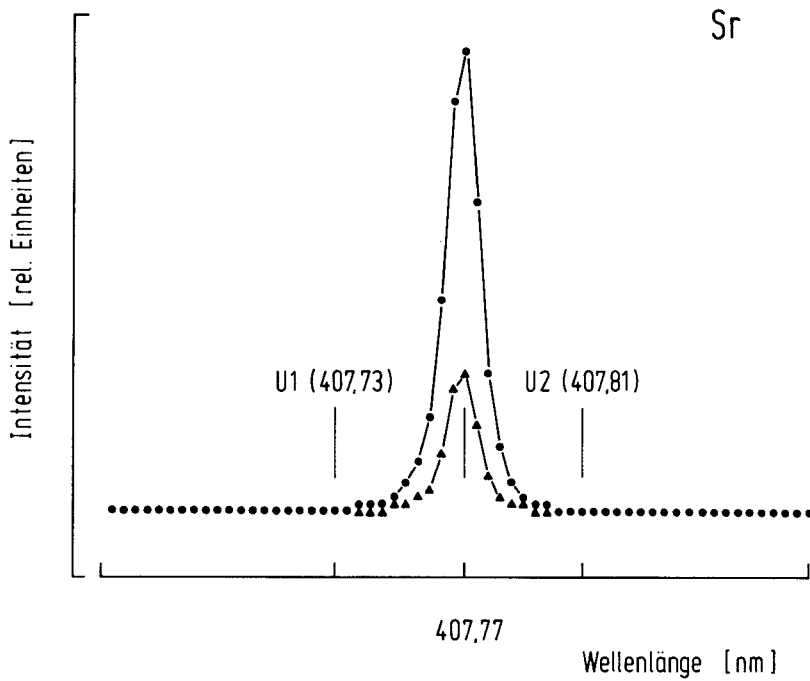
Fig. 20. Sr-spectra in human urine (▲) and standard addition of 100 µg/L Sr (●).
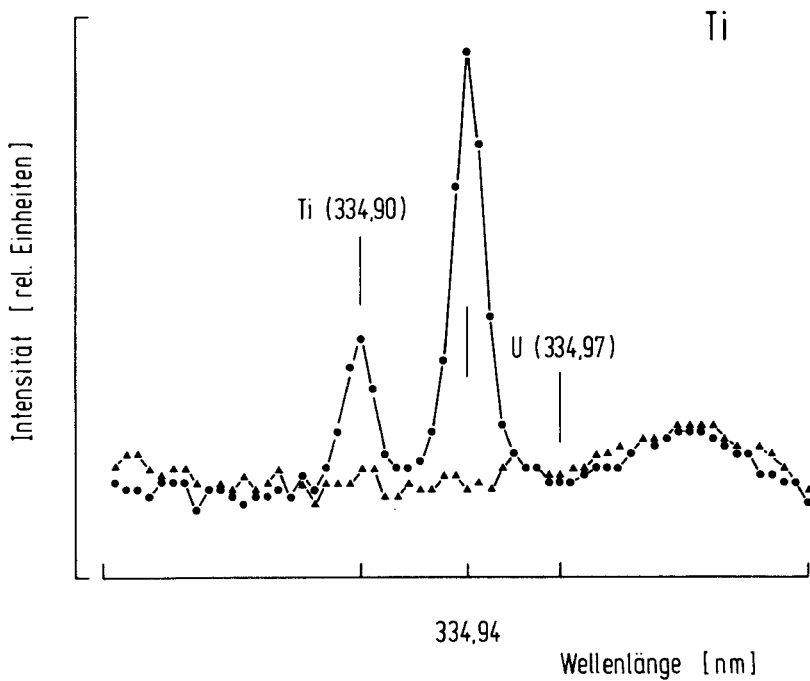
Fig. 21. Ti-spectra in human urine (▲) and standard addition of 10 g/L Ti (●).

TABLE 8

TYPICAL CONCENTRATION OF TRACE ELEMENTS IN MUSCLE, LIVER AND KIDNEY (STANDARD REFERENCE MATERIALS; BCR)

Element	Bovine Muscle (mg/kg dry weight)		Bovine Liver (mg/kg dry weight)		Pig Kidney (mg/kg dry weight)	
Cu	2.36 ±	0.06	189 ±	4	31.9 ±	0.4
Zn	166 ±	3	142 ±	3	128 ±	3
Fe	79 ±	2	214 ±	5	299 ±	10
Mn	334 ±	28 [µg/kg]	9.3 ±	0.3	8.5 ±	0.3

In actual "damage to forest" ("acid rain"- problems) investigations, the main matrices which must be analyzed are needles, leaves, pieces of wood, roots and soil. The elements Al, B, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, S, Sr, Ti, V, and Zn can be determined routinely in such samples by ICP/DCP-spectrometry (Table 9). The concentrations of the heavy metals Cd, Pb, Hg, As and others are in most cases too low (exception: soil, for these analytical techniques). A similar picture can be obtained for the field of bioindicators for environmental influences (plants, animals, organs of man) (Schramel et al., 1984; Wolf et al., 1984) (Table 10). In case of soils and sludges, in most the samples one can obtain all relevant elements, such as the legally regulated heavy metals Cd, Pb, Zn, Cu, Ni, and Cr (Table 11,12) (Schramel et al., 1982).

Especially for the determination of Pb and Cd, it is necessary to take into account possible interelement interferences (e.g. Al→Pb; Fe→Cd) which may lead to systematic errors. A critical evaluation of the spectra is necessary for finding the adequate background correction position and other parameters as well. The expenditure which must be done is again dependent on the spectral resolution power of the instrument used for the analysis.

In this connection, one has to point out again the necessity and the importance of adequate standard reference materials (SRM). Adequate here means similar matrix and similar concentration ranges to the samples which should be analyzed. Using these

TABLE 9

TYPICAL VALUES FOR TRACE ELEMENT CONCENTRATIONS IN SPRUCE-NEEDLES DETERMINED BY ICP-AES (IN mg/kg DRY WEIGHT)

Al	Ca	Cu	Fe	K	Mg	Mn	Ni	P	S	Sr	Ti	Zn
50	350	3.0	75	8500	780	560	1.5	2500	1700	1.4	1.5	30

TABLE 10

CONCENTRATIONS OF TRACE ELEMENTS IN DIFFERENT "BIOINDICATORS" (mg/kg DRY WEIGHT) DETERMINED BY ICP-AES

Elements (mg/kg)	Moss (n = 250)	Snails (n = 170)	Earthworms (n = 50)	Soils (different types) (n = 130)
Cu	4.5 - 250	20 - 600	8 - 200	5 - 100
Zn	25 - 450	60 - 550	100 - 900	20 - 150
Mn	15 - 1100	25 - 1000	50 - 700	200 - 1000
Ti	2.5 - 550		50 - 400	100 - 600

TABLE 11

CONCENTRATIONS OF DIFFERENT ELEMENTS IN TYPICAL SLUDGE-SAMPLES (mg/kg DRY WEIGHT) DETERMINED BY ICP-AES. COMPARISON OF "AQUA REGIA" AND HF (TOTAL)-DIGESTION

Sample	Be	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Ti	V	Zn
Aqua Regia											
sludge 1	0.30	20	104	420	8100	220	40	330	150	16	2500
sludge 2	0.45	82	770	990	16000	560	294	1240	280	25	3700
sludge 3	0.30	5	460	820	43000	430	950	490	88	14	2800
total (HF)											
sludge 1	0.85	20	115	440	8000	260	42	330	1900	40	2700
sludge 2	5.5	83	775	1000	16500	650	330	1260	20000	260	4000
sludge 3	0.65	5.2	550	820	44000	460	1120	495	1200	33	3200

TABLE 12

CONCENTRATIONS OF DIFFERENT ELEMENTS IN TYPICAL SOIL-SAMPLES (mg/kg DRY WEIGHT) DETERMINED BY ICP-AES AFTER "AQUA REGIA" DIGESTION

Sample	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Ti	V	Zn
soil 1	0.12	10.5	5.9	8630	236	8.3	13.2	160	11.8	19.0
soil 2	0.30	26.5	201	17470	935	20.5	19.3	325	27.2	97
soil 3	0.22	30.4	10.3	24105	1080	19.9	15.3	208	28.7	47.7

materials, one can test the final determination method to avoid systematic errors. One is not allowed to check the whole analytical procedure (especially drying and mineralization steps) by using a SRM, because e.g. volatile compounds of trace elements may have disappeared from the SRM during its preparation, but are present in the original samples. SRM's may only be used for testing the final step of the analytical determination, especially the right choice of the emission line (wavelength) for an undisturbed measurement and for the adequate position for background correction.

CONCLUSION

Plasma emission spectrometry, especially ICP- and DCP-sources, has a fixed place in modern trace element analysis. In spite of the relatively small number of relevant elements detectable by these techniques for the biomedical and environmental fields of application, plasma emission spectrometry can deliver a lot of possibly important information. The main advantages are the multielement character of the technique (sequentially or simultaneously), nearly chemical interference free measurements, control of physical interferences, a relatively high level of accuracy and precision, high specificity, fast multielement determinations (especially in case of a simultaneous device), low sample consumption and in general a wide range of detectable elements (Table 13).

Present efforts to improve the performance of the method are based especially on the development of new sample introduction systems: new types of pneumatic nebulizers (Mc Kinnon et al., 1981; Layman and Lichte, 1982), some injection techniques for reducing the sample consumption (Aziz et al., 1981; Broekaert and Leis, 1979; Kimberley et al., 1984; Greenfield, 1983), ultrasonic nebulization (Goulden and Anthony, 1984; Mermet et al., 1981; Taylor and Floyd, 1981), electrothermal evaporation (Gunn et al., 1978; Kirkbright and Snook, 1979; Millard et al., 1980; Nixon et al., 1974), direct sample insertion (Kirkbright and Walton, 1982; Li-Xing et al., 1983; Salin and Horlick, 1979; Sommer and Ohls, 1980), hydride generation (Broekaert and Leis, 1980; Fry et al., 1979; Thompson et al., 1978) which offers limits of detection for e.g. As and Se in the order of about 1 μg/L and direct solid sampling techniques (Aziz et al., 1984; Broekaert et al., 1985; Human et al., 1976). The development of low power and low gas consumption torches (Allemand et al., 1979; Rezaaijaan and Hieftje, 1985; Van der Plas and De Galan, 1984) promises a significant reduction of the relatively high operating costs. A new generation of data acquisition and data handling systems for all the spectral information promises further improvements precision and diminishing of bias.

TABLE 13

CAPACITY OF ICP-AES FOR TRACE ELEMENT ANALYSIS IN THE BIO-MEDICAL AND ENVIRONMENTAL FIELD (DATA FOR THIS PAPER TAKEN FROM MEASUREMENTS WITH A SEQUENTIAL ICP-SPECTROMETER JY 38, INSTRUMENTS S.A., FRANCE)

Element	Wavelength (nm)	Signal/background (Xmg/L)		Detec.limits (3σ) (μg/L)		Possible line overlapping from (in bio-medical and environmental samples)	Relevance for		Detec. limit for sufficient		Remarks	RF-power for aqueous solutions (this paper)
		Winge et al. (1984)	this paper	Winge et al. (1984)	this paper		Biomed.	environment	Biomed.	environment		
Ag	328.068	38 (10)	—	7	—	Mn	—	—(?)	—	—(o)	(?o) rel. high content in sludges,	
Al	396.152	10.5 (10)	10 (10)	28	13	Mo,Zr	+	+	o	+	1	900 W
As	193.696	56 (100)	—	53	—		+	+	—	—		
Au	242.795	170 (100)	—	17	—	Mn,Sr	—?	—?	—	—(o)	(?o) rel. high content in sludges,	
B	249.773	63 (10)	20 (10)	5.7	7.0	Fe	+?	+	+	+		1.0kW
Ba	455.403	230 (10)	42 (1)	1.2	0.35	Cr	—	—	+	+		900 W
Be	313.042	110 (1)	—	0.27	0.2	(17)	+?	+	o	o	(17)	800 W
Bi	223.061	87 (100)	—	34	—		—	—	—	—		
C	247.856	17 (100)	—	176	—		+	+	o	o	total carbon	900 W
Ca	393.366	178 (1)	130 (1)	0.19	0.55		+	—	+	+		
Cd	214.438	12 (1)	5.5 (1)	2.5	2.9	Fe	+	+	—	o(+)		1.0 kW
Ce	413.765	62 (100)	—	48	—		—	—	—	—		
Co	238.892	50 (10)	23 (10)	6	7.8		+	—	—	—		
Cr	267.716	42 (10)	30 (10)	7.1	6.0	V	+	+	—	o(−)	1	900 W
Cs	452.673	7 (100000)	—	42000	—		—	—	—	—		

TABLE 13 (continued)

Element	Wavelength (nm)	Signal/background (Xmg/L)		Detec.limits (3σ) (µg/L)		Possible line overlapping from (in bio-medical and environmental samples)	Relevance for		Detec. limit for sufficient		Remarks	RF-power for aqueous solutions (this paper)
		Winge et al. (1984)	this paper	Winge et al. (1984)	this paper		Biomed.	environment	Biomed.	environment		
Cu	324.754	56 (10)	40 (10)	5.4	3.0	OH-band, Mn, Mo	+	+	+(o)	+		900 W
Dy	353.170	30 (10)	—	10	—		—	—	—	—		
Er	337.271	29 (10)	—	10			—	—	—	—		
Eu	381.967	110 (10)	—	2.7	—		—	—	—	—?		
Fe	259.940	48 (10)	32 (10)	6.2	2.5	Ta,,Mo	+	+	+	+	1	900 W
Ga	294.364	64 (100)	—	46	—		—	—	—	—		
Gd	342.247	21 (10)	—	14	—		—	—	—	—		
Ge	209.426	75 (100)	—	40	—		—	—	—	—		
Hr	277.336	190 (100)	—	15	—		—	—	—	—		
Hg	197.227	120 (100)	—	25	—		+	+	—	—(o)		
Ho	345.600	53 (10)	—	5.7	—		—	—	—	—		
In	230.606	47 (100)	—	63	—		—	—	—	—		
Ir	224.268	110 (100)	—	27	—		—	—	—	—		
K	766.490	— —	—	42857	100		+	—	+	0		
La	394.910	75 (10)	—	4	—		—	—	—	—(o)		
Li	460.286	3.5 (100)	—	857	—		+	—	—	—		
Lu	261.542	150 (5)		1	—		—	—	—	—		
Mg	279.553	195 (1)	70 (1)	0.15	0.18	Fe,Cu	+	+(—)	+	+		1.0 kW
Mn	257.610	220 (10)	18 (1)	1.4	1.4		+	+	+(o)	+		900 W
Mo	281.615	21 (10)	14 (10)	7.9	9.3		+	+	—	—(o)	1	900 W
Na	588.995	101 (100)	4.2 (1)	29	2.9		+	+	+	+		1.0 kW
Nb	309.418	83 (100)	—	36	—		—	—	—	—		

Nd	401.225	59 (100)	—	50	—		—	—	—	—		
Ni	231.604	19 (10)	20 (10)	15	10	Co	+	+	—	−(o)	1	900 W
Os	225.585	83 (1)	—	0.36	—		—	—	—	−(?)		
P	213.618	39 (10)	—	76	40		+	+	o	+(o)	total phosphorus	900 W
Pb	220.353	70 (100)	—	42	67		+	+	—	−(o)		
Pd	340.458	68 (100)	—	44	—		—	—	—	—		
Pr	390.844	81 (10)	—	37	—		—	—	—	—		
Pt	214.423	100 (100)	—	30	—		—	—	—	—		
Rb	420.185	0.8 (100)	—	37500	—		+(?)	+(?)	—	—		
Re	197.313	49 (10)	—	6	—		—	—	—	—		
Rh	233.477	67 (100)	—	44	—		—	—	—	—		
Ru	240.272	100 (100)	—	30	—		—	—	—	—		
Sb	206.833	91 (100)	—	32	—		+	+	—	—		
Sc	361.384	200 (10)	—	1.5	—		—	+(?)	—	−(o)		
Se	196.026	40 (100)	—	75	—		+	+	—	—		
Si	251.611	250 (100)	—	12	—		+	+	o	o		
Sm	359.260	69 (100)	—	43	—		—	—	—	—		
Sn	187.980	120 (100)	—	25	—		+	+	—	−(o)		
Sr	407.771	72 (1)	54 (1)	0.42	0.11	Cu,Sn,Ta,Mo,Cr	−(?)	+(?)	+	+		1.0 kW
Ta	226.230	120 (100)	—	25	—		—	−(?)	—	−(o)		
Tb	350.917	130 (100)	—	23	—		—	—	—	—		
Te	214.281	73 (100)	—	41	—		—	—	—	—		
Th	283.730	48 (100)	—	65	—		—	−(?)	—	—		
Ti	334.941	79 (10)	11 (1)	3.8	2.3	Cu,Co,Au,W	+(?)	+	−(o)	+		
Tl	351.924	15 (100)	10 (100)	200	174	Fe	−(?)	+	—	—		1.0 kW
Tm	313.126	58 (10)	—	5.2	—		—	—	—	—		
U	385.958	12 (100)	—	2.50	—		—	−(?)	—	—		
V	307.311	60 (10)	—	5	10		+	+	—	−(o)		1.0 kW
W	207.911	100 (100)	—	30	—		—	−(?)	—	—		

TABLE 13 (continued)

Ele-ment	Wave-length (nm)	Signal/background (Xmg/L)		Detec.limits (3σ) (μg/L)		Possible line overlapping from (in bio-medical and environmen-tal samples)	Relevance for		Detec. limit for sufficient		Remarks	RF-power for aqueous solutions (this paper)
		Winge et al. (1984)	this paper	Winge et al. (1984)	this paper		Biomed.	environ-ment	Biomed.	environ-ment		
Y	371.030	86 (10)	—	1.8	—		—	—	—	—		
Zn	213.856	170 (10)	52 (10)	1.8	3.4	Ni,Fe,Cu	+	+	+	+		900 W
Zr	343.823	42 (10)	—	7.1	—		—	—	—	—		

Relevance: + + very important, + important, ? unknown, − not important;
detection limit: + + very sufficient, + sufficient, o adequate, − not sufficient;
remarks: 1 the most sensitive line was not taken for the measurements.

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Chapter 6

Voltammetry

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INTRODUCTION

The objective of this discussion is to introduce the analyst to the potential of voltamme-
tric techniques for trace element analysis of body fluids. Electroanalytical methods, and
particularly voltammetric ones, are probably the most widely used competition for spec-
troscopy in trace metal determinations. Voltammetry is a major branch of electroanalysis
in which a potential is imposed upon an electrochemical cell and the resulting current is
measured. The electrochemical cell contains the sample solution (usually of 5-50 mL
volume) into which the working, reference and counter electrodes are immersed. The
potential of the working electrode is varied in some systematic manner to cause electroac-
tive chemical species to be reduced or oxidized. The current flowing is a direct measure of
the rate of this redox reaction. The resulting current-potential curve, known as voltammo-
gram, exhibits a peak-shaped, or sigmoidal (wave-like), response over the potential region
where the analyte undergoes the electron-transfer (redox) reaction. The exact shape of
the response depends on the potential program used. The magnitude of the limiting (or
peak) current is proportional to the concentration of the analyte, and is used to provide
the quantitative information (usually in conjunction with a calibration plot, standard addi-
tions or internal standards). The position (potential) of the peak, or wave, serves to
identify the species. A large variety of working electrodes have been used with voltam-
metry. In the special case where the working electrode is the dropping mercury electrode
(DME), the technique is known as polarography, and the current vs. potential curves are
called polarograms. Understanding redox processes is essential for sound application of
voltammetric techniques. Familiarity with basic electrochemistry and the principles of vol-
tammetry is prerequisite. Elementary and advanced discussions are found in various
monographs (Kissinger and Heineman, 1984; Bond, 1980; Wang, 1985; Plambeck, J.A.,
1982).

WHY VOLTAMMETRY?

The application of voltammetry is wide and versatile. With the development of advanced stripping and pulse techniques, voltammetric methods become highly competitive with other instrumental methods of analysis in terms of sensitivity and reliability. Numerous studies have indicated that modern voltammetric techniques are capable of determining metals accurately at trace and ultratrace levels, often with multielement capability and inexpensive instrumentation. Multipurpose, reliable and robust commercial instruments are available for a relatively modest investment of $15,000. The instrumentation has a low power demand and does not have any special installation requirements (cooling, ventilation, etc.). Voltammetric analysis covers, with its various schemes, a very wide concentration range (from the upper mg/L level down to ng/L). Since this approach is based on Faraday's law, it provides high inherent reliability (bias and precision). For example, an interlaboratory comparison of various techniques used for blood lead analyses, conducted by the Center of Disease Control, ranked stripping voltammetry first based on its overall precision and accuracy (Boone et al., 1979). Besides applications for routine clinical determinations, voltammetry is to be regarded a a cross-check method for clinical laboratories using atomic absorption spectrometry. Another advantage, that may be exploited more in the near future, is the ability of voltammetry to provide information regarding the distribution (i.e., speciation) of a given metal. Recent advances in electrode technology, particularly the development of chemically modified electrodes and ultra-microelectrodes further enhance the power of voltammetry. There is no doubt, based upon the above points, that voltammetry deserves much broader recognition for day-to-day trace metal analysis in the clinical laboratory. It is the author's opinion, as well as many others (e.g., Bersier, 1987), that many people are not well aware of the special features and power of modern voltammetry. The discrepancy between the potential feasibility and the actual clinical application of voltammetry may be attributed to reasons other than education and tradition. A disadvantage of voltammetry, compared to various atomic absorption procedures, is the requirement for a tedious sample pretreatment (decomposition) step. In addition, voltammetry does not apply to as wide a range of metals as its spectrometric competitors. New voltammetric approaches based upon flow injection analysis and metal-chelate adsorptive accumulation address properly the speed and scope limitations of voltammetry, and enhance its competitive power for clinical measurements.

In the following sections, the characteristics of the most commonly applied voltammetric methods for measurements of trace metals will be discussed, together with their applicability to analysis of clinical samples, modes of operation and pretreatment procedures.

STRIPPING ANALYSIS

Because of its remarkable sensitivity (down to 10-11 M) stripping analysis is the most widely used voltammetric technique for trace metal determination in clinical samples (Wang, 1982A). Stripping analysis can be considered as a two-step technique. In the first step, metals in solution are effectively preconcentrated onto the working electrode by electrodeposition:

$$M+n + ne- + Hg \longrightarrow M(Hg) \tag{1}$$

The deposition potential is usually several tenths of a volt more negative than the formal potential of the least easily reduced ion to be determined. The longer the precon-centration period, the lower the detection limit. Small- volume mercury electrodes (hanging mercury drop or mercury film), are commonly employed as working electrodes. The mercury film electrode has a higher surface-to-volume ratio than the hanging mercury drop electrode and consequently yields lower detection limits. The preconcentration step is usually facilitated by convective transport (solution stirring or flow, or electrode rotation) to enhance the flux of the metal ions to the surface. The reduced elements are re-oxidized in the second (measurement) step by means of a positive-going linear or pulse potential ramp, and the resulting stripping current is monitored. The order in which the metals are stripped from the electrode is a function of the formal potential of the metal. The peak current is proportional to the bulk concentration of the metal (C), as well as to the electrode area (A), preconcentration time (t), and the flux of the metal ion at the surface during preconcentration (m):

$$ip = kACtm \tag{2}$$

It is thus essential for the various parameters of eq. 2 to be as reproducible as possible to ensure that good precision is obtained. Hence, qualitative and quantitative information is obtained by measuring the peak potential and peak current, respectively. The concentra-tion is determined by standard additions or a calibration curve. Simultaneous analysis of up to five elements may be done. Figure 1, for example, shows typical voltammograms for three metals of clinical concern (cadmium, lead, and copper), at the parts-per-billion level, following 5-min preconcentration and using the linear scan and differential pulse stripping modes. These voltammograms were obtained at a mercury-coated microelectrode (of 280μm diameter) that is highly suitable for stripping analysis of very small sample vol-umes. (The use of even smaller electrodes made of carbon fiber allows multicomponent trace analysis of solutions of 5 μL volume (Baranski and Quon, 1986).) Conventional stripping analysis can be used to determine about 20 metals that are electrolytically deposited and/or form amalgam with mercury (eq. 1). Examples of metals which have been determined at mercury electrodes are lead, cadmium, gallium, copper, thallium, antimony, bismuth, zinc, indium, or tin. Other metals, such as selenium, mercury, arsenic, platinum, gold, or silver have been determined by analogous stripping measurements at
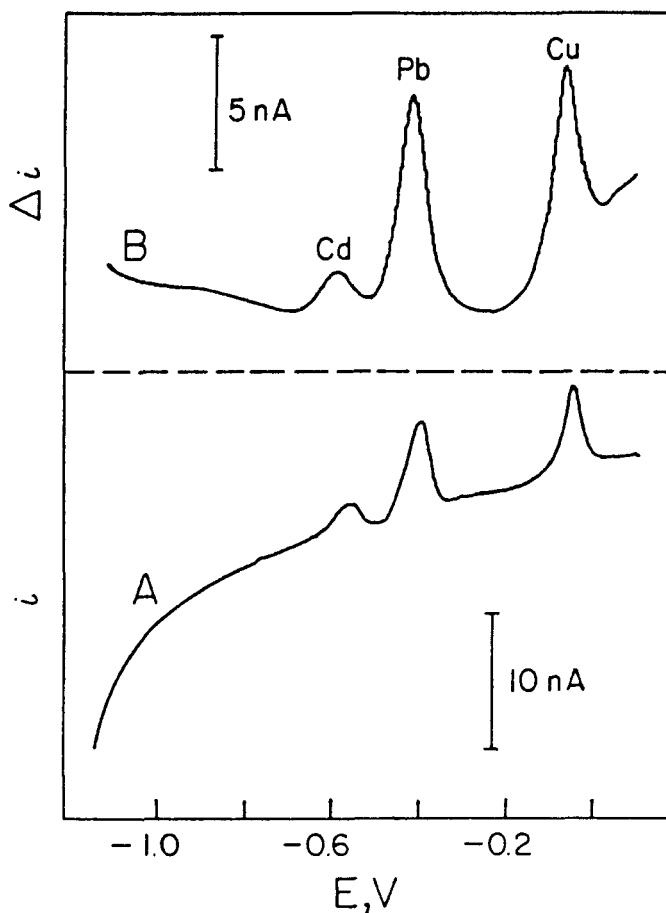
Fig. 1. Characteristic stripping voltammograms for 1.2 x 10^{-8} M cadmium, 2.5 x 10^{-8} M lead, and 7.5 x 10^{-8} M copper. Five min deposition at -1.15 V; electrolyte, 0.1 M KNO_3. (A) linear scan mode; (B) differential pulse mode. (From Wang, 1982B).

bare solid electrodes (e.g. gold film, glassy carbon). Thus, the technique does not apply to as wide a range of metals as does atomic spectroscopy. Overlapping peaks, formation of intermetallic compounds and surfactant adsorption at the mercury surface are the main types of interferences in stripping analysis. The extent of these depends upon the specific sample and experimental conditions. There are many variants of the basic technique useful for expanding its scope, enhancing its sensitivity and for minimizing interferences. Those variants relevant to clinical analysis are discussed in the following sections. A useful description of the technique with a comprehensive survey of applications is available (Wang, 1985).

Potentiometric Stripping Analysis

Potentiometric stripping analysis (PSA) resembles conventional stripping voltammetry in that trace metal analytes are electrolytically deposited in a thin film of mercury. However, after this preconcentration, the potentiostatic circuitry is disconnected and the amalgamated elements are chemically (rather than electrochemically) oxidized by reaction with an excess of a suitable oxidant:

$$M(Hg) \xrightarrow{\text{oxidant}} M + n \tag{3}$$
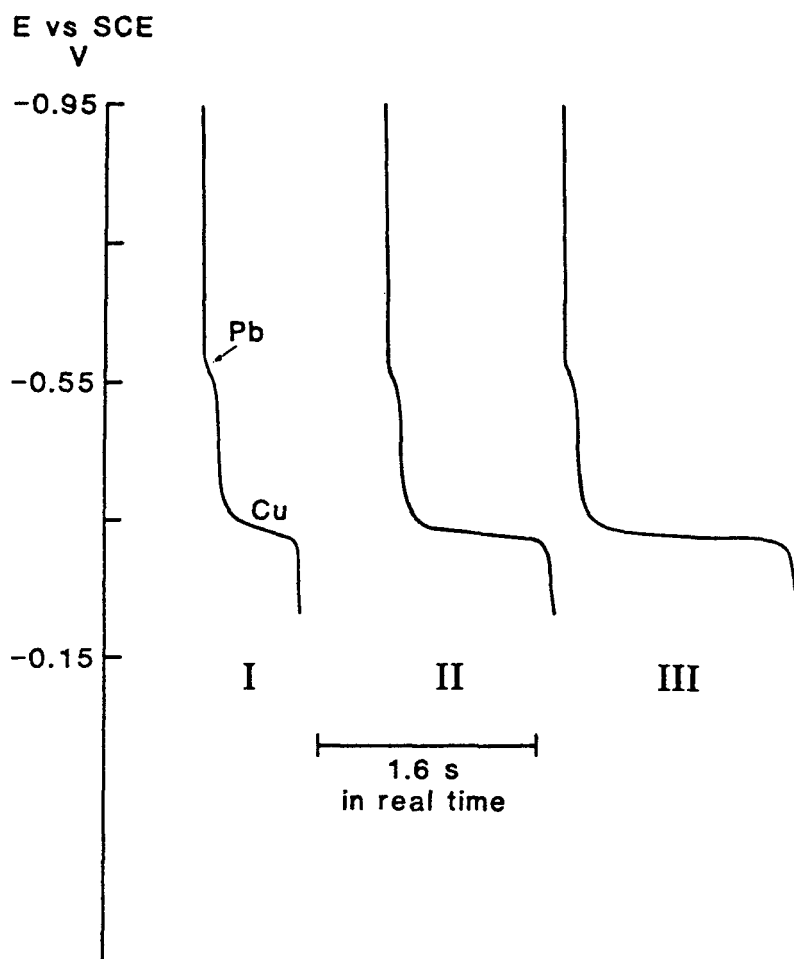
Fig. 2. Potentiometric stripping analysis in a whole blood sample. Deposition for two min at -1.2 V (Curve I). Curves II and III are the corresponding response after standard additions of 8 and 16 μM copper, respectively. (From Jagner et al., 1981).

The potential of the working electrode, when monitored as a function of time, gives an experimental curve which is similar to a redox titration curve. A sharp potential change accompanies depletion of the metal from the mercury. The time needed for completing the oxidation (i.e., to reach the equivalence point) is proportional to the concentration metal ion in solution. For example, Figure 2 shows potentiometric stripping curves for copper in a dilute whole blood sample following 2-min preconcentration (curve I) and the response after standard additions of 8.0 and 16 μM copper (curves II and III). A more convenient peak-shaped response (of dt/dE vs. E) is provided by modern PSA instrumentation (through derivatization of the sigmoidal signal). Since no current is drawn through the working electrode during the stripping step, this approach does not suffer from interferences from other electroactive species which might be present in the sample (Jagner, et al., 1981). PSA is applicable not only to heavy metals but also to highly electropositive elements (Coetzee et al., 1983). For example, sodium and potassium ions can be measured in blood serum after adding an excess of dimethyl sulfoxide. Other features of PSA have been reviewed (Jagner, 1982).

Adsorptive Stripping Voltammetry

Many elements of clinical significance cannot be plated electrolytically or form amalgam with mercury, and thus can not be determined by conventional stripping voltammetry. Alternative schemes based on nonelectrolytic preconcentration of the metal have been developed recently to extend the scope of stripping voltammetry toward these elements. In particular, adsorptive stripping voltammetry has recently made rapid progress and additional metals can now be conveniently determined down to the parts-per-trillion concentration level. This approach involves the formation of an appropriate surface-active chelate of the metal, its controlled adsorptive accumulation on the hanging mercury drop electrode, and the voltammetric quantitation of the surface-bound species usually by a negative-going potential scan. The voltammetric response is directly proportional to the surface concentration, with the adsorption isotherm providing the relationship between the surface and bulk concentrations of the chelate. To enhance the sensitivity, optimum conditions for maximum chelate adsorption are employed during the preconcentration step. These developments have led to effective procedures for measuring low concentrations of nickel, iron, cobalt, molybdenum, manganese, chromium, or vanadium in the presence of the complexing ligands dimethylglyoxime, solochrome violet RS, nioxime, oxine, eriochrome black T, diethylenetriamine pentaacetic acid or catechol, respectively. Short preconcentration periods (2-4 min) are sufficient for convenient measurements at the nanomolar concentration level. Deviations from linearity may be observed around the micromolar level, as full surface coverage is approached. Interferences from co-existing surfactants (that compete on adsorption sites) are eliminated by UV irradiation. Additional information on the adsorptive approach can be found in recent review articles (Wang, 1989, van den Berg, 1991). Lower detection limits (down to picomolar level) can be achieved by coupling the interfacial accumulation with catalytic effects (Wang et al., 1992B). Nonelectrolytic accumulation of trace metals prior to their voltammetric quantita-

tion can be accomplished by another promising avenue involving electrodes modified with an appropriate preconcentrating agent (ligand, ion-exchanger, etc.) (Baldwin et al., 1986).

PULSE POLAROGRAPHY

In cases where the concentration of an element approaches the micromolar level, the use of highly sensitive stripping procedures is not necessary. In this case, faster pulse polarographic procedures can be used. The purpose of using pulse polarography is to discriminate against the major component of the background response, the charging current, and hence to determine concentration of dilute solutions (down to 10-8 M). Hence, pulse techniques are much more sensitive than conventional (dc) polarography. Pulse polarography takes advantage of the fact that, following a sudden change in applied potential, the charging current decays much more rapidly than does the diffusion-controlled current of interest. By the time the current is sampled (ca. 40 ms after the pulse), the charging current contribution is negligible. Typical waveforms which have been employed include the staircase, differential pulse, normal pulse, and square wave.

In the most common version of pulse polarography, differential pulse polarography (DPP), a series of short potential pulses are superimposed on a linear potential ramp (with one pulse applied for each mercury drop) (Figure 3A). Most often, the pulses have a height of 50 mV and a duration of 60 ms. The total current is measured just before the pulse is applied and is measured again late in the pulse life. When the difference between these readings is plotted as a function of the applied potential, a peak-shaped voltammogram is generated. In this way, it is possible, for example, to measure As(III), over a wide concentration range, down to 3x10-9 M (Osteryoung, 1983). Arsenic(V), the other oxidation state, does not interfere. While DPP effectively corrects for the charging current contribution, it suffers from the relatively long time required for recording the voltammogram (typically 2 to 4 min).

Another powerful (and much faster) approach for the charging current problem is square wave polarography. The potential waveform employed is that obtained by superimposing a symmetrical square wave on a staircase waveform (Figure 3B). The current is sampled twice during each square cycle, once at the end of the forward pulse and again at the end of the reverse pulse. The difference between these two measurements is plotted against the staircase potential to yield current-potential peaks with height proportional to the bulk concentration. Fast scan rates (up to 1 V/s) can be employed; thus, the complete voltammogram is recorded in a matter of seconds (during the life of a single mercury drop). This speed enhancement (compared to DPP) requires no sacrifice of sensitivity. Such characteristics are very attractive for a clinical laboratory performing a lot of tests. The application (and advantages) of square wave polarography for the rapid and reliable determination of zinc, cadmium, lead, copper, nickel, and cobalt in biological samples has been illustrated (Ostapczuk et al., 1986). With the recent introduction of commercial instruments capable of performing the technique, this approach is expected
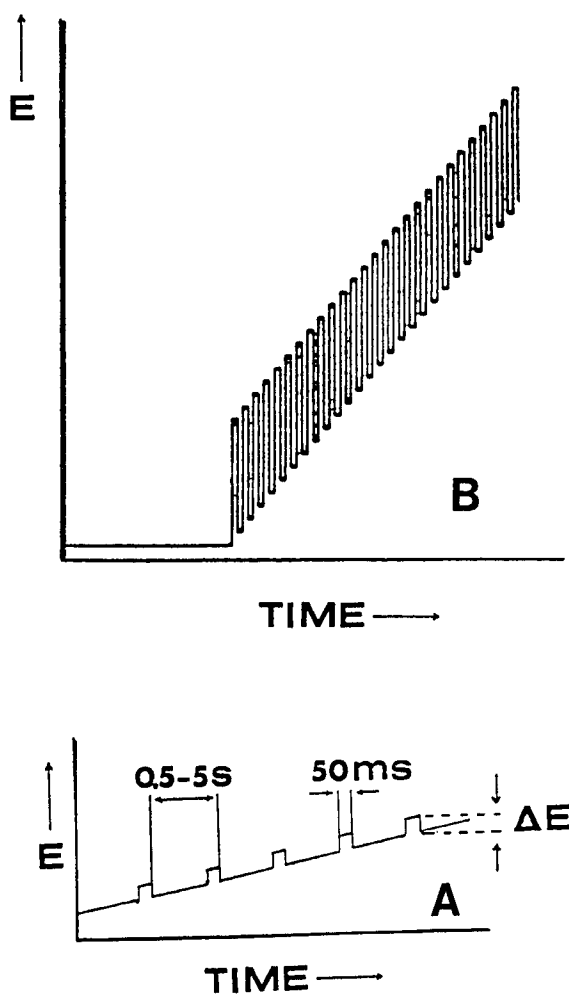
Fig. 3. Potential-time waveforms used for: (A) differential pulse polarography; (B) square-wave polarography.

to find a widespread use in the clinical laboratory. For a more complete description of the pulse polarographic techniques see Flato (1972).

Table I summarizes the properties of voltammetric methods for trace metal analysis, including comparison of their sensitivity.

TABLE I

CHARACTERISTICS OF VOLTAMMETRIC METHODS FOR TRACE METAL ANALYSIS

Technique[a]	Working Electrode[b]	Detection Limit, M	Speed (time per cycle), min
DC Polarography	DME	10^{-5}	3
DP Polarography	DME	10^{-8}	3
SW Polarography	DME	10^{-8}	0.1
Stripping Voltammetry	HMDE, MFE	10^{-10}	3-6
Adsorptive Stripping Voltammetry	HMDE	10^{-10}	2-4
Adsorptive Catalytic Stripping Voltammetry	HMDE	10^{-12}	2-4

[a] DC = direct current; DP = differential pulse; SW = square wave
[b] HMDE = hanging mercury drop electrode; MFE = mercury film electrode

AUTOMATED VOLTAMMETRIC ANALYSIS

The advent of inexpensive computing power has changed dramatically the way voltammetric determinations are performed. In particular, automated multielement determination schemes and high speed flow systems are now available for convenient and effective trace metal determination in biological samples.

Adeloju et al. (1985) developed a simple and reliable multielement approach for the determination of up to eight elements in biological materials. This approach relies on a range of voltammetric techniques (pulse and stripping). Figure 4, for example, shows the scheme employed for sequential simultaneous determination of selenium, cadmium, lead, copper, zinc, nickel, and cobalt in a single sample solution. Excellent precision and accuracy were demonstrated by the use of standard reference materials. Speed and convenience were obtained by using microprocessor-controlled instrumentation. The use of the static mercury drop electrode readily enabled change from a polarographic mode to a stripping mode (and vice versa). Other possible combinations of metals that can be determined in a single sample solution using the proposed analytical scheme are summarized in Table II.

The coupling of voltammetric analysis with rapid flow injection systems can be used to improve the efficiency and versatility of trace metal determinations. In particular, flow injection stripping systems are attractive for clinical determinations since they increase substantially the assay rate and require small sample volumes, while maintaining high sensitivity and multielement capability (Wang, 1983; Jagner, 1983). For example, Frenzel and Bratter (1986) described the direct determination of cadmium and lead in acid digests of biological samples using injection rates of up to 200 h^{-1}. Further advantages can be obtained by the convenient use of the medium-exchange approach that minimizes various
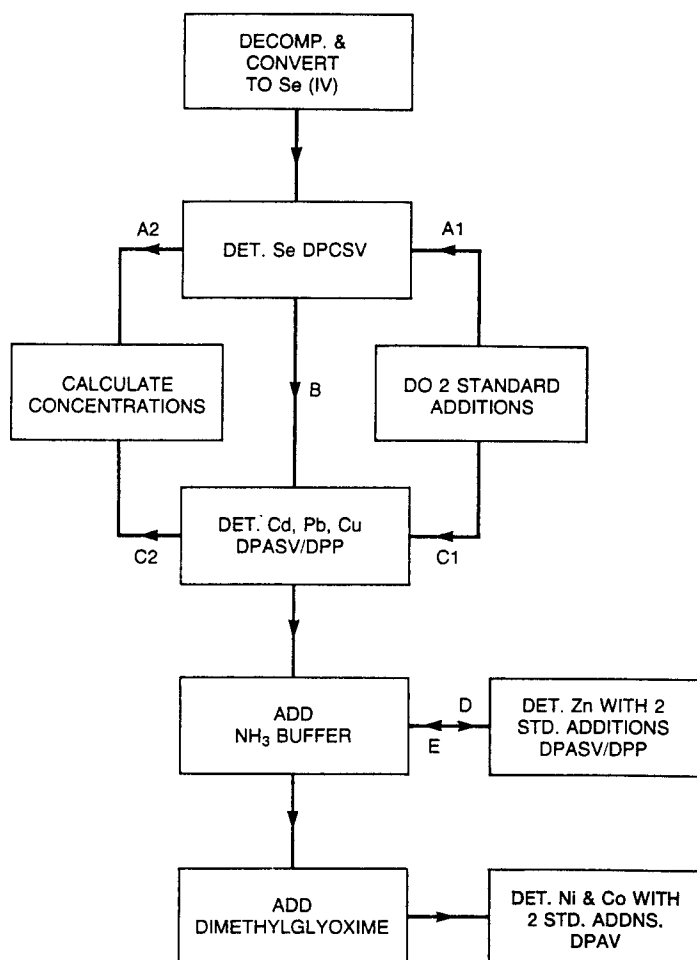
Fig. 4. Analytical scheme for sequential simultaneous determination of selenium, cadmium, lead, copper, zinc, nickel, and cobalt in a single sample solution. (From Adeloju et al., 1985).

matrix interferences (because the stripping step is performed in a solution of optimum composition, irrespective of sample composition). Being a closed system, contamination problems are greatly reduced. Commercial electrochemical flow detectors, e.g., thin layer or wall-jet cells, are usually employed in these experiments.

An automatic system, that couples the speed of square-wave polarography with a control of the sample handling is available commercially (Yarnitzky, 1985). Further reduction in assay time is achieved using a nebulizer to eliminate the time-consuming deaeration step. Microprocessor-based voltammetric analyzers are available from companies such as EG&G PAR, Metrohm, Bioanalytical Systems, ECO Chemie or Tacussel.

TABLE II

COMBINATIONS OF ELEMENTS MEASURABLE BY THE MULTIELEMENT VOLTAMMET-
RIC SCHEME IN A SINGLE SAMPLE

Elements determined sequentially	No. of elements	Range of techniques utilized[a]	Total analysis time[b], min
Se, Cu	2	DPCSV, DPP	55
Cu, Ni	2	DPP, AdSV	30
Se, Cu, Zn	3	DPCSV, DPP	65
Se, Co, Ni	3	DPCSV, AdSV	70
Cu, Co, Ni	3	DPP, AdSV	40
Se, Cu, Co, Ni	4	DPCSV, DPP, AdSV	80
Cu, Zn, Co, Ni	4	DPP, AdSV	50
Se, Cd, Pb, Cu	4	DPCSV, DPASV, DPP	90-115[c]
Cd, Pb, Cu, Ni, Co	5	DPASV, DPP, AdSV	90-115[c]
Se, Cd, Pb, Cu, Zn	5	DPCSV, DPASV, DPP	105-130[c]
Se, Cd, Pb, Cu, Ni, Co	6	DPCSV, DPASV, DPP, AdSV	120-145[c]
Cd, Pb, Cu, Zn, Ni, Co	6	DPASV, DPP, AdSV	105-130[c]
Se, Cd, Pb, Cu, Zn, Ni, Co	7	DPCSV, DPASV, DPP, AdSV	135-160[c]
As, Cd, Pb, Cu, Zn, Ni, Co	7	DPCSV, DPASV, DPP, AdSV	135-160[c]

[a] DPCSV = differential pulse cathodic stripping voltammetry; DPP = differential pulse polarography; AdSV = adsorptive stripping voltammetry; DPASV = differential pulse anodic stripping voltammetry.
[b] Includes deoxygenation time and two standard additions.
[c] Depends on concentration of cadmium in sample; analysis time can be more if $\ll 1$ μg/L or less if > 5 μg/L.
(From Adeloju et al., 1985)

SPECIATION STUDIES

The potential of voltammetric techniques for trace metal speciation is recognized to be of utmost importance in biomedical research. Measurement of the total concentration of a trace element provides no information about its toxicity and/or its role in the body. Important speciation information can be achieved in voltammetric measurements on the basis of the observed shifts in the potential of the voltammetric peak (or wave) of metal ions in the presence of complexing agents, based on their ability to distinguish between oxidation states of a given element in solution, and the discrimination between labile (i.e., reactive) and inert (i.e., unreactive) metal species (Florence, 1986). Besides speciation information obtained from direct voltammetric analysis, additional knowledge can be obtained by coupling voltammetry with various preliminary treatments of the sample. For example, the ability to measure only complexes with a reasonable rate of ligand exchange was exploited for determining zinc-amino acid complexes in body fluids following a dialysis step (Martins and Johansson, 1980). A study combining stripping voltammetry with gel filtration

chromatography resulted in a new knowledge of the interaction and time distribution of heavy metals in the serum and red blood cells (Griffin and Matson, 1972). Information about kinetic stability and stoichiometry of various metal-protein complexes could be obtained using titrimetric stripping procedures or plotting pseudostripping voltammograms (plots of peak current vs. deposition potential). The process of uptake and transport of a trace metal by a biomembrane resembles the electrodeposition step in stripping analysis (Florence, 1986). The interaction of metals with biosurfaces can be evaluated via a proper choice of the experimental conditions. (Because voltammetric speciation data are operationally-defined, it should be possible to select experimental conditions so that the kinetics of electrodeposition are similar to the rate of uptake of a trace metal by a biological system.) While most applications of voltammetry for speciation analysis have been concerned with the distribution of heavy metals in environmental matrices, future efforts will undoubtedly focus at analogous analyses of body fluids.

SAMPLE PRETREATMENT FOR VOLTAMMETRIC ANALYSIS

In applications of voltammetry to biological samples, it is often the sample rather than the sensitive voltammetric analyzer that is the limiting factor. Getting the sample into a form that can take full advantage of the instrument capability may be the hardest part of the analysis. For this reason, the sample is usually treated prior to analysis. Such treatment releases the trace metals bound to sample components, and minimizes fouling of electrode (by adsorption of certain sample components) or background currents (from other electroactive constituents). The precision and bias of the data obtained by voltammetric analysis of biological samples will be more dependent on how well the sample is decomposed than with many other analytical techniques (e.g., atomic absorption spectroscopy which relies on atomization of the metal from the solution).

Solid samples (e.g., tissue, bone, teeth) must be decomposed. This is often the case with blood samples. With voltammetric techniques there appears to be an increasing tendency to use wet digestion methods. For example, reliable stripping analysis of human hair or teeth have been reported following digestion with nitric acid (Chittleborough and Steel, 1980; Oehme et al., 1978). Wet digestion of blood is usually carried out by boiling an aliquot of the blood specimen with a mixture of nitric acid, sulfuric acid, and perchloric acid. This approach may suffer from high background (contamination) originating from the glassware and acids used. This problem is more serious in determinations of cadmium because of the low concentration of this metal in blood. Obviously, judicious precautions must be taken to minimize the contamination problem. Due to the risk of explosion that always exists when organic matter is digested with acid, proper safety precautions must be followed. To minimize this danger, a perchloric acid-free digestion was suggested (employing a mixture of sulfuric and nitric acids followed by a treatment with hydrogen peroxide (Peter and Reynolds, 1976). Wet digestion procedures often employ highly diluted blood samples (up to 1:50), which do not allow measurement of metals (e.g., cadmium) present at the nanomolar concentration level in the original sample. Lund and

Eriksen (1979) *compared different wet digestion procedures for voltammetric stripping* analysis of urine and preferred the use of a mixture of nitric, sulfuric, and perchloric acids. Satisfactory recovery (92-100%) of lead and cadmium from spiked samples was obtained. An effective procedure based on a rapid (20 min.) digestion of freeze-dried urine samples coupled with voltammetric analysis was reported (Golimowski et al., 1979).

Dry ashing pretreatment methods offer lower blank levels than wet digestion, and are thus desirable for trace measurement. The use of these methods for accurate determination of trace metal in biological samples by voltammetric techniques requires careful consideration of the acid employed for the dissolution of the ash and the possible effect of the carbonaceous residue. Adeloju et al. (1984) evaluated several dry ashing methods for the simultaneous stripping determination of cadmium and lead in biological materials. Dry ashing methods are generally unsuitable for the voltammetric determination of selenium and arsenic because of losses of these elements at the high temperature.

The use of the Metexchange reagent provides the simplest and fastest pretreatment scheme for voltammetric analysis (Morrell and Giridhar, 1976). The reagent − consisting of 1.1% $CrCl_3$, 1.4% Ca-acetate and spike of Hg(II) − rapidly decomplexes (via an ion-exchange substitution reaction) trace metals contained in the sample. It has been particularly useful for reliable and rapid voltammetric determination of lead in blood. The lead content can be determined with an analysis cycle of about 90 s per sample. Main limitations are the high price of the reagent and the large dilution factor (1:29). Other schemes for pretreating biological samples in conjunction with voltammetric analysis have been reported. Low energy UV or high energy γ irradiations were used to decompose the organic matter and release the bound metals of blood samples (Batley and Farrar, 1978). This approach has the advantages of low risk of contamination and minimal sample manipulation.

Direct assays following sample dilution, without any prior treatment, have been employed in various situations. For example, potentiometric stripping analysis that is less prone to background interferences has been shown useful for the determination of cadmium and lead in whole blood (Jagner et al., 1981; Ostapczuk, 1992). Sample dilution or pH adjustment were shown useful for stripping measurements of thallium, copper, cadmium, and lead in urine (Franke et al., 1976; Lund and Eriksen, 1979). Although these procedures worked successfully, they involve the general risk of unreliability. More reliable is the *direct stripping voltammetric determination of lead in urine after a simple clean-up* involving removal of organic compounds on a Sep-Pak cartridge (Bond and Reust, 1984). The filtration step may also be performed in-situ at the electrode surface, using a permselective polymeric coating such as cellulose acetate (Wang and Hutchins-Kumar, 1986) or Nafion (Hoyer and Florence, 1987). Also attractive is the use of fumed silica to remove interferences by urinary organic surfactants (Stauber and Florence, 1990).

SELECTED APPLICATIONS OF VOLTAMMETRY

The following section describes selected studies, representing the potential of voltammetry for determining trace metals in biological samples.

Franke and De Zeeuw (1976) described a stripping procedure for systematic screening of ten heavy metals in urine. Qualitative identification was based on measuring the peak potentials in different electrolyte solutions and working electrodes. Complexing agents were used to improve the selectivity, e.g., EDTA for masking lead and cadmium that interfere in thallium measurements. Acid-digested urine samples were analyzed for mercury using potentiometric stripping analysis (Jagner and Aren, 1982). A flow system with a medium-exchange capability and a gold working electrode were employed. Deposition conditions include 90 s period and -0.25 V potential. An effective reduc- tillation-stripping procedure for the measurement of total arsenic in blood and urine was described (Davis et al., 1978). Ashed materials were subjected to a reductillation procedure to reduce As(V) to As(III) and to separate arsenic from the sample matrix. The method is rapid (12 samples per hour), low bias (6%), and reproducible. Blood and urine samples were analyzed for trace amounts of bismuth by addition of hydrochloric acid, separation by ion-exchange chromatography and determination by stripping voltammetry (Kauffmann, 1981).

DeAngelis et al. (1977) combined the light sensitivity of stripping voltammetry with the small sample volume (70 μL) advantage of a thin-layer cell for rapid and reliable measurements of the lead content of human blood. Each analytical cycle was performed within 60 s. Good precision and low bias were reported. Sample droplets can also be analyzed for their lead content in connection with disposable (screen-printed) electrodes (Wang and Tian, 1992). Such electrodes are expected to play a major role in mass screening programs in the U.S.A., aimed at preventing lead poisoning in children. Ostapczuk (1992) described a simple and rapid potentiometric procedure for determining lead and cadmium in whole blood. The applicability of anodic stripping voltammetry and graphite furnace atomic absorption spectrometry to the determination of antimony in blood and urine was compared (Costantini et al., 1985). Although the bias and precision were comparable, the voltammetric approach allowed measurement of lower concentrations and rapid calibration with aqueous solutions; the GF-AAS scheme was faster. A useful cathodic stripping procedure for the determination of selenium in whole blood and serum was reported (Bi-xia, 1985). The method employed wet digestion and a preconcentration potential of -0.5 V, and yielded a detection limit of 0.1 parts-per-billion and recovery of 91-108%.

Alternating current polarography was employed in a rapid, simple and sensitive assay of lead in bones (Ladanyi and Stadler, 1984). The bone samples were solubilized and oxidized with a HNO_3-$HClO_4$ mixture; the acids were evaporated and the residue was dissolved in the supporting electrolyte solution. No interference from the high inorganic salt concentrations (from the bone matrix) was observed. The sensitivity was 2 mg/L, with reliable measurements possible up to 1000 mg/L. A reliable procedure for measuring nickel in human nails based on adsorptive stripping voltammetry of the nickel-dimethylglyoxime complex was reported (Gammelgaard and Andersen, 1985). A simple wet

digestion procedure was employed. The voltammetric approach offered ca. 100-fold lowering of the detection limit compared to atomic absorption spectroscopy, resulting with much more reliable data for low levels of nickel. A 2-6% deviation from the true value was reported. Adsorptive stripping voltammetry has been used for reliable determination of platinum in human blood (Nygren et al., 1990). The method involved a dry ashing treatment followed by the adsorptive-catalytic reduction of the platinum-formazone complex. Good reproducibility and accuracy were reported also in the determination of zinc, cadmium, lead, and copper in human hair, following digestion with nitric acid, fusion with alkali metal nitrates and anodic stripping quantification (Chittleborough and Steel, 1980). Anodic stripping voltammetry was used also, in conjunction with an acid digestion bomb, for determining bismuth, thallium, zinc, lead, and cadmium in teeth, feces, hair, and urine, for medical management and post-mortem studies (Kinard, 1977). Further examples of the use of voltammetry for analysis of body fluids may be found elsewhere (e.g., Wang, 1985). In conclusion, voltammetry has many inherent advantages as an analytical technique, in particular high sensitivity and reliability. It should enjoy greater popularity in clinical analysis than it does presently. An increased use of voltammetry for large scale-routine applications in analytical toxicology and surveillance of trace metals in man is expected, based on the diverse examples (given above) and the recent advances in the field of voltammetry (methodology and portable instrumentation). The relationship between voltammetry and spectroscopic techniques in practical biomedical applications should be complementary rather than competitive.

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Chapter 7

Neutron activation analysis

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INTRODUCTION

The history of neutron activation analysis goes back to the middle of the 1930s when it was first described by G. Hevesy and H. Levi at the Niels Bohr Institute in Copenhagen. The principle of the technique is that elements can be made radioactive by exposure to neutron irradiation. Two types of physiological processes are associated with this activation: one prompt and one delayed. Classically, neutron activation analysis is based on the detection of the delayed event, viz. the characteristic radiation emitted during the decay — with a specific half-life ($t_{1/2}$) — of the unstable nuclei formed by (n,γ) reaction.

Over the years, the fundamental principles of the technique remained essentially unchanged but remarkable developments in detection instrumentation and electronic equipment continuously enlarged its applicability. Thus, neutron activation started as a single-element technique when investigators had only Geiger-Müller counters and NaI(Tl) detectors at their disposal; with the development of high-resolution Ge(Li) detectors or, more recently, high-purity (HP) Ge detectors and associated multichannel analyzers, it became a really multielement technique. Currently, neutron activation analysis may be regarded as an extremely powerful analytical technique with excellent sensitivity for many (though not all) elements for which all sources of systematic or random variation are identifiable and predictable down to the limits of detection.

The three main sources of neutrons for irradiation are nuclear reactors, radioactive neutron sources, and electron and ion accelerators which produce high-energy neutrons; the first give the highest neutron fluxes and permit the highest sensitivities for detection and quantification of various elements (down to the 10^{-9}-10^{-12} g and less). This chapter only deals with reactor neutron activation analysis — the other sources will not be considered further. Similarly, in vivo techniques will not be treated.

Nuclear reactors consist of uranium or uranium oxide, enriched in ^{235}U, dispersed in a moderator of graphite, water, or deuterium oxide which slows down the fission (or fast) neutrons (energy, or E, > 1 MeV, M = mega, eV = electron volt) to epithermal (1 MeV > E > 0.5 eV) and thermal (E < 0.05 eV) neutrons.

This chapter was conceived more as a source of general information and further reading rather than as a practical expedient for the use of neutron activation analysis in a laboratory. Readers in search of the latter information are referred to other publications (Adams et al., 1971; Bowen, 1981; Erdtmann and Petri, 1986; Guinn and Hoste, 1980; Heydorn, 1984; and Versieck, 1988).

FUNDAMENTALS

During irradiation, samples are exposed to thermal, epithermal, and fission neutrons. The distribution at the site of the sample in the reactor depends on the type of the moderator and the distance traveled by the neutrons. The type of the nuclear reaction and its cross section varies with the energy of the neutrons. The absorption of thermal and epithermal neutrons lead to (n,γ) reactions, e.g., ^{1}n + ^{55}Mn → ^{56}Mn + γ or ^{55}Mn(n,γ)^{56}Mn, ^{1}n + ^{63}Cu → ^{64}Cu + γ or ^{63}Cu(n,γ)^{64}Cu, and ^{1}n + ^{65}Cu → ^{66}Cu + γ or ^{65}Cu(n,γ)^{66}Cu; the presence of fission neutrons gives rise to threshold reactions (vide infra).

At low neutron energies, the cross sections of many nuclides are inversely proportional to the neutron velocity (1/v law, v = velocity). Cross sections for mono-energetic neutrons at a velocity of 2200 m·s^{-1} (the most probable velocity in a Maxwellian distribution for 20°C), corresponding to an energy of 0.0253 eV, are generally indicated by σ_0; effective cross sections or "average thermal cross sections", measured in a well-thermalized reactor whose conventional flux is known by determination with an 1/v detector, are commonly represented by σ_{th}. Up to approximately 0.5 eV, deviations from the 1/v law for most reactions are so small that the differences are within the bias of the measurements so that σ_0 and σ_{th} can be substituted for one another. Above 0.5 eV, reaction cross sections no longer follow the 1/v law. The wide energy region from 0.5 eV to 1 MeV is referred to as the resonance region because many isotopes show high cross sections (so-called resonance peaks) occurring at discrete neutron energies in this region. To calculate reaction rates, an effective cross section I_0 or resonance integral has been defined. The element most frequently used to determine epithermal neutron fluxes and to calibrate other resonance integrals is gold.

If a sample contains N nuclei of a particular stable isotope, the rate of formation of its (n,γ) product nuclei is N$\Phi\sigma$ in which Φ is the neutron flux density of the thermal and epithermal neutron flux density in n·cm^{-2}·s^{-1} (Φ = Φ_{th} + Φ_{epi}) and σ the (n,γ) cross section (generally expressed in barns, one barn equalling 10^{-24} cm^2) of the target nuclei in the neutron spectrum in which the sample is irradiated ($\sigma = \sigma_{th}\cdot\Phi_{th}/\Phi + I_0\cdot\Phi_{epi}/\Phi$).

If the (n,γ) product is unstable, nuclei will decay at a rate of λN^*, λ being the radioactive-decay, first-order rate constant of the radioisotope (ln 2/$t_{1/2}$ or 0.693/$t_{1/2}$) and N* the

number of the radioactive nuclei which are present. Thus, at any given time during a steady irradiation, the net rate of the formation of radioactive product nuclei equals $N\Phi\sigma - \lambda N^*$. After integration, the basic neutron activation analysis equation is written as follows:

$$A_0 = N\Phi\sigma(1 - e^{-\lambda t_i}) \tag{1}$$

A_0 is the activity in disintegrations per second, (in becquerel - Bq) exactly at the end of the irradiation (at zero decay time, $t_d = 0$) and t_i is the time of irradiation. The expression in parentheses, $1 - e^{-\lambda t_i}$, is called the saturation factor or S. It varies from 0 to 1: zero for $t_i = 0$ or an extremely short irradiation, one for $t_i \gg t_{1/2}$ or an infinitely long irradiation relative to the $t_{1/2}$ of the radioisotope, In practice, S rapidly and asymptotically approaches the limiting value of one with increasing t_i relative to the $t_{1/2}$ of the activated radioisotope species. At saturation, A_0 simply equals $N\Phi\sigma$ (steady state of formation of the radioisotope, rate of formation equaling rate of decay).

The tern N in the basic neutron activation analysis is equal to $w\Theta N_A/AW$, in which w is the weight of the element, Θ the percent isotopic abundance of the target nuclide, N_A Avogadro's number, and AW the atomic weight of the element. Thus, the basic equation can be rewritten as follows:

$$A_0 = \frac{w\Theta N_A}{AW} \Phi\sigma S \tag{2}$$

In practice, equations (1) and (2) are rarely used. When a sample and a standard are irradiated in identical conditions and counted on the same detector, the much simpler equation may be applied:

$$\frac{A'_0(\text{sample})}{A'_0(\text{standard})} = \frac{w(\text{sample})}{w(\text{standard})} \tag{3}$$

where A'_0 (sample) and A'_0 (standard) are the counting rates (counts per second, cps) of sample and standard and are equal to εA_0 (sample) and εA_0 (standard), where ε is the detection efficiency of the detector. Since w (standard) — the weight of the element in the standard — is known, w (sample) — the weight of the element in the sample — is easy to calculate.

Under routine experimental conditions, it is not necessary to use A'_0 (sample) and A'_0 (standard). Indeed, after irradiation the induced radioactivity decreases exponentially according to the $t_{1/2}$ of the radioisotopic species: the decay factor (D) is given by $e^{-\lambda t_d}$, t_d being the time between the end of the irradiation and the moment of the measurement. Thus, since $A_t = A_0 \cdot e^{-\lambda t_d}$, the comparator equation (3) can also be written for any stated decay time, t_d, after the end of the irradiation:

$$\frac{A'_{t_d}(\text{sample})}{A'_{t_d}(\text{standard})} = \frac{w(\text{sample})}{w(\text{standard})} \tag{4}$$

Since sample and standard cannot be counted on the same detector at the same time, an adjustment must be made according to the equation:

$$A'_{t'_d} = A'_{t_d} \cdot e^{-\lambda(t'_d - t_d)} \tag{5}$$

The above described equations apply to all the numerous nuclear reactions giving rise to radionuclides and almost all the elements present in the sample become activated.

Instead of co-irradiating a standard for each element to be determined, preference may be given to the application of a comparator method (De Corte et al., 1969; De Corte et al., 1987; Girardi et al., 1965; and Moens et al., 1984).

As mentioned earlier, fission or fast neutrons five rise to threshold reactions of the type (n,p), (n,α), and (n,2n). With Z = atomic number, the first and the second lead to the production of respectively, Z-1 and Z-2 radionuclides, e.g., ^{56}Fe(n,p)^{56}Mn, ^{65}Cu(n,p)^{65}Ni, ^{31}P(n,α)^{28}Al, or ^{54}Fe(n,α)^{51}Cr. Although the cross sections (average fast neutron cross sections or σ_f) for these reactions are low compared to those for thermal neutron activation, serious interferences may ensue.

NUCLEAR DECAY

Radioactive nuclei, formed by neutron activation, disintegrate by beta-, gamma-, or — exceptionally — alpha-emission; in biomedical neutron activation analysis, only the first and the second are of practical importance.

Under beta-disintegration are classified types of nuclear decay in which the atomic number changes whereas the mass number of the nucleus stays unchanged. In the process, the radioactive nucleus emits a β^- (negatron) (atomic number increases by one unit) or a β^+ particle (positron) (atomic number decreases by one unit). The emitted beta rays show a broad energy spectrum starting at about zero, extending up to a finite maximum energy, E_{max}, which is characteristic of the nuclide, and having an intensity maximum, e.g., at approximately $E_{max}/3$ as in the spectrum of ^{64}Cu. Because of their continuous nature, beta spectra of different isotopes are difficult to individualize and are, therefore, very rarely used for quantitative measurements. Electron capture (EC) (capture of an orbital electron by the nucleus) is also a type of beta disintegration. The obtained daughter isotope has an atomic number one unit less than the parent isotope (but a practically unchanged nuclear mass) and can be de-excited by gamma decay; in case of electron capture, one also always has a characteristic X-ray due to orbital electron rearrangement in the daughter nucleus. A radioactive isotope can decay to several excited states or to a ground state by means of several corresponding β^-, β^+, or EC processes.

Gamma rays are monoenergetic, electromagnetic radiations: mass and charge of gamma-emitting nuclei thus remain unchanged. Gamma emission frequently accompanies beta decay and carries away the energy of an excited level to the ground state nucleus or to another excited state of lower energy. Gamma emission also results from isomeric

transition (IT) in cases where the (n,γ) products of the irradiation are metastable nuclear isomers, e.g., ^{60m}Co, ^{69m}Zn, ^{77m}Se, ^{86m}Rb, and several others. Isomeric transition is free from accompanying beta radiation.

In a number of instances, radioisotopes decay to radioactive daughter substances which are very suitable nuclides for counting: well-known examples are ^{99}Mo ($t_{1/2}$ = 66.02 h or hours) which decays to ^{99m}Tc ($t_{1/2}$ = 6.02 h, gamma line = 140.5 keV or kiloelectronvolt), ^{115}Cd ($t_{1/2}$ = 53.46 h) which decays to ^{115m}In ($t_{1/2}$ = 4.486 h, gamma line = 336.2 keV), and ^{113}Sn ($t_{1/2}$ = 115.09 d or days) which decays to ^{113m}In ($t_{1/2}$ = 1.658 h, gamma line = 391.7 keV).

RADIOCHEMICAL VERSUS INSTRUMENTAL PROCEDURES

In radiochemical neutron activation analysis (RNAA), separations are applied; in instrumental neutron activation analysis (INAA) no chemistry is involved – samples are irradiated and counted over a period of time to obtain the desired information about the elements of interest. For most elements, spectral interference-free gamma rays are available and where there are two isotopic species at the same energy, their contribution can generally be computed.

In biomedical trace element research, radiochemical separations are mostly applied to eliminate matrix activities. Indeed, all biological matrices contain large amounts of sodium, chlorine, and potassium: they become highly radioactive upon irradiation so that their photopeaks swamp the energies of other isotopes of interest. The half-lives of ^{24}Na (causing the most serious difficulties), ^{38}Cl, and ^{42}K being, respectively, 14.949 h, 37.21 min, and 12.36 h, problems are mainly associated with the determination of elements via short-lived radioisotopes: vanadium (^{52}V, $t_{1/2}$ = 3.75 min), manganese (^{56}Mn, $t_{1/2}$ = 2.5785 h), copper (^{64}Cu, $t_{1/2}$ = 12.701 h), zinc (^{69m}Zn, $t_{1/2}$ = 13.76 h), arsenic (^{76}As, $t_{1/2}$ = 26.32 h), molybdenum (^{99}Mo, $t_{1/2}$ = 66.02 h; ^{101}Mo, $t_{1/2}$ = 14.6 min), cadmium (^{115}Cd, $t_{1/2}$ = 53.46 h) and mercury (^{197}Hg, $t_{1/2}$ = 64.1 h). For all these elements, separations were developed at the University of Ghent (Cornelis et al., 1981; Versieck et al., 1973; Versieck et al., 1974; Versieck and Vanballenberghe, 1985; Versieck et al., 1978; and Versieck et al., 1990). When elements may be determined via long- lived radioisotopes as in the case of chromium (^{51}Cr, $t_{1/2}$ = 27.69 d), cobalt (^{60}Co, $t_{1/2}$ = 5.271 y or years), zinc (^{65}Zn, $t_{1/2}$ = 244.0 d), selenium (^{75}Se, $t_{1/2}$ = 119.770 d), rubidium (^{86}Rb, $t_{1/2}$ = 18.66 d), and cesium (^{134}Cs, $t_{1/2}$ = 2.062 y), the above mentioned matrix activities may simply be allowed to decay so that they no longer present a serious problem (Versieck et al., 1977). However, a new problem emerges: indeed, ^{32}P ($t_{1/2}$ = 14.28 d, which is formed by (n,γ) reaction from ^{31}P), produces an intense Bremsstrahlung which prevents the determination of a long-lived but low-energy radioisotope such as ^{51}Cr – this is the reason why it has to selectively separated in cases of plasma or serum analysis (Versieck et al., 1978). Mercury poses a special problem because the unique 279.2 keV gamma ray photopeak of ^{203}Hg coincides with the 279.5 keV gamma ray photopeak of ^{75}Se.
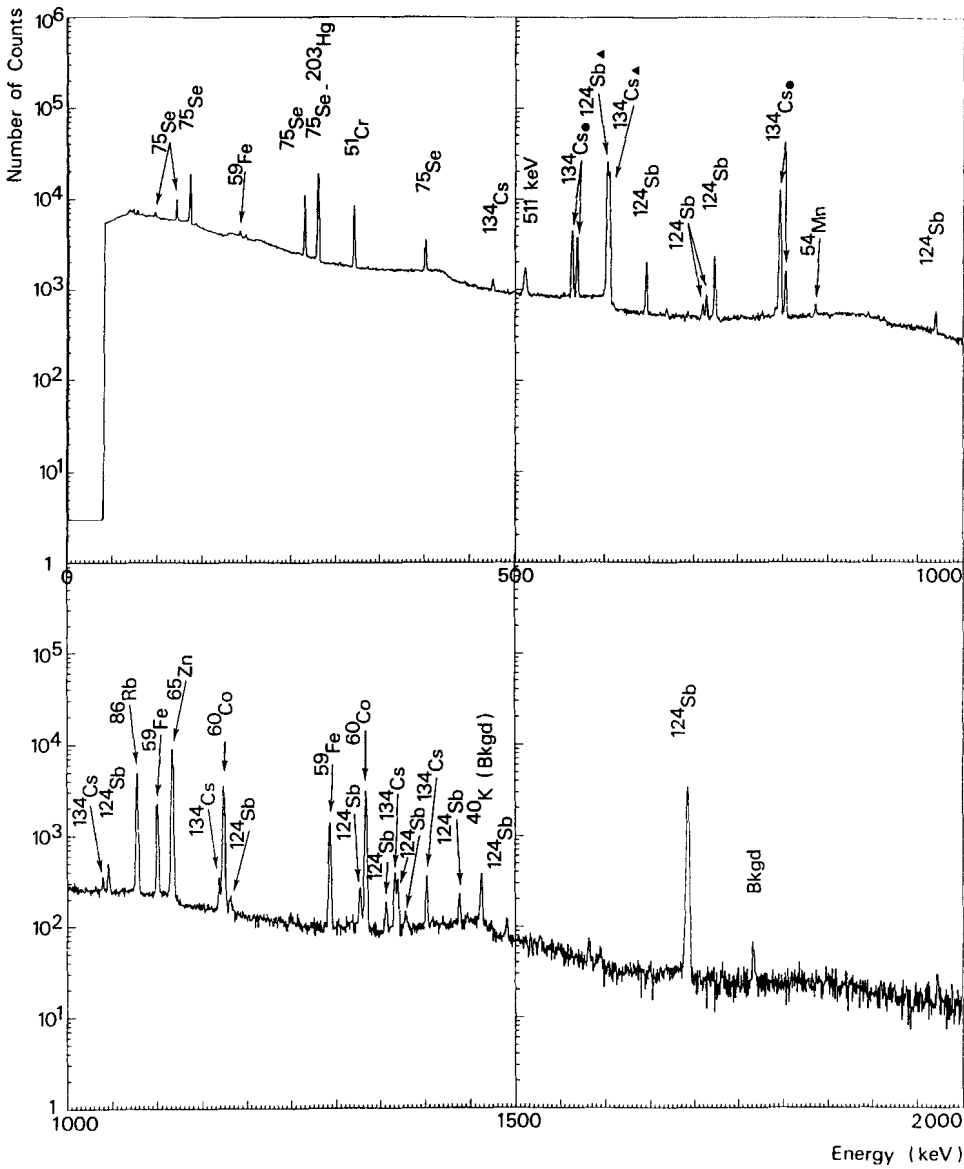
Figure 1

COUNTING EQUIPMENT

When a radioisotope is separated from an activated sample (for example by solvent extraction, distillation, precipitation, or adsorption on inorganic ion exchangers or ion-exchange resins) it can be counted using a simple, cheap beta or gamma counter; in other instances, more sophisticated measurement systems are needed.

For simple gamma counting, thallium-activated sodium iodide [NaI(Tl)] scintillation detectors, which became commercially available in the early 1950s, continue to render excellent services. In spite of the introduction of many other scintillation materials, they remained preeminent. NaI(Tl) detectors can be manufactured in various sizes and shapes — characteristics which play an important role in counting efficiency. Well-type detectors have a higher efficiency but a lower resolution than cylindrical detectors. Because of the limited energy resolution, peaks observed in a pulse-height spectrum from a NaI(Tl) detector are very broad. Nevertheless, the routinely available resolution of 7.5 - 8.5% at the 661.6 keV ^{137}Cs photopeak is sufficient to discriminate a few photopeaks under proper conditions. So, NaI(Tl) detectors have been employed in connection with multi-channel analyzers of 256-400 channels, e.g., for the determination of manganese, copper, and zinc in serum and packed blood cells in the author's laboratory in the early 1970s (Versieck et al., 1973, Versieck et al., 1974). They continue to be used because of their robustness and good detection efficiency, and their reasonable price.

For truly multielement determinations, increased selectivity is required. It is offered by semiconductor detectors, e.g., by lithium-drifted germanium [Ge(Li)] or intrinsic germanium (high-purity) [HP-Ge] types with a resolution — full width at half maximum or FWHM — of approximately 1.0 keV at 122 keV (^{57}Co) and approximately 1.8 keV at 1332.5 keV (^{60}Co). It must be noted that the outstanding improvement in energy resolution when compared to NaI(Tl) detectors is obtained at the price of the overall detection efficiency (approximately 25-30% relative to a 3" x 3" NaI(Tl) crystal for large, common sizes of Ge(Li) or HP-Ge detectors. Germanium detectors are, inevitably, many times more expensive than their sodium iodide counterparts. To make efficient use of their high resolution, (expensive) multichannel spectrometers with some 4000 channels are needed; usually, they include electronic components, such as a pile-up rejector, a percentage deadtime meter, a preset timer, and an oscilloscope display. The system is calibrated with radioisotope sources of two or more gamma rays of exactly known energies.

Fig. 1 (on p. 152). HP-Ge spectrum of a multielement standard (serum doped with chromium, iron, cobalt, zinc, selenium, rubidium, antimony, and cesium) irradiated 7 times for approximately 7 h at a neutron flux of $1.77 \cdot 10^{12}$ n·cm^{-2}·s^{-1} and counted for 12 h, 18 days after the end of the irradiation. The figure illustrates the excellent resolution of modern radioactivity counting equipment. As soon as their energies differ by some 5 keV, photopeaks are sufficiently resolved to allow the calculation of their number of counts as in the case of the 563.2 and 569.3 keV and the 795.8 and 801.9 keV gamma ray photopeaks of ^{134}Cs (indicated by ı). Only when their energies differ only by 2 or 3 keV, they largely overlap as exemplified by the 602.7 keV and 604.7 keV photopeaks, respectively of ^{124}Sb and ^{134}Cs (indicated by s).

The excellent resolution obtained with the radiation detection system, described in the preceding paragraphs, is illustrated in Figure 1 which shows a HP-Ge spectrum of a multielement standard (serum doped with chromium, iron, cobalt, zinc, selenium, rubidium, antimony, and cesium) irradiated 7 times for approximately 7 h at a neutron flux of $1.77 \cdot 10^{12}$ n·cm^{-2}·s^{-1} and counted for 12 h, 18 d after the end of the irradiation. The excellent resolution of the system is illustrated by the individualization of the numerous photopeaks with energies between 500 and 1500 keV (channel numbers 1000-3000, the spectrometer being operated at a gain setting of 0.5 keV per channel). As soon as their energies differ by 5 or 6 keV (e.g., the 563.2 and 569.3 keV and the 795.8 and 801.9 keV photopeaks of ^{134}Cs) (indicated by ●), photopeaks are sufficiently resolved to allow peak integration (calculation of the net counts of the peak) with reasonable reliability; when their energies differ only by 2 or 3 keV, they largely overlap as illustrated by the 602.7 keV photopeak of ^{124}Sb and the 604.7 keV photopeak of ^{134}Cs (indicated by ▲).

The large amount of data generated with Ge(Li) or HP-Ge radiation detector systems makes it very useful to have a computer for information processing. The equipment can be programmed to compare the areas of specified peaks in the spectra of samples and standards and, thus, to calculate the elemental levels. For additional information, the reader is referred to publications focusing on analysis of gamma spectra and reduction of data (Herpers, 1986; Op de Beeck and Hoste, 1976; Perlman, 1981; and Yule, 1981).

SENSITIVITY

It is generally agreed that neutron activation analysis has shown great sensitivity for many elements. Absolute sensitivities of detection depend on the atomic weight of the element, the fractional abundance of the target nuclide, and its cross section for thermal neutrons (which are fixed values) as well as on the available neutron flux, the irradiation time, the decay period, and the counting efficiency of the detector (which are variable parameters). The formulae described under Fundamentals (vide supra) will make it clear that, unless conditions are exactly specified, published values cannot easily be compared especially as the definitions for sensitivity chosen by the investigators may be different. Experimental sensitivities may be idealized because of matrix problems, difficulties in radiochemical separations, and other analytical problems associated with the analysis of complex, real samples.

Guinn and Hoste (1980) published a series of "best detection sensitivities" — arbitrarily defined as the amount of the element (in μg) that gives 30 net counts under the photopeak — in the absence of interfering activities using the gamma ray peak of the (n,γ) product of the element providing the most sensitive detection of the element determined for $\Phi = 10^{13}$ n·cm^{-2}·s^{-1}, $t_i = 5$ h, $t_d = 0$, $t_c = 100$ min, a 40 cm^3 Ge(Li) detector, and a sample-to- detector distance of 2 cm. For detailed information, the reader is referred to the original publication. Table 1 shows the values for some of the most interesting elements and their most frequently used (n,γ) radioisotopes. In spite of their obvious limitations, figures give a useful indication of the capability of neutron activation analysis to

TABLE 1

CALCULATED APPROXIMATE LIMITS OF DETECTION[a] FOR BIOLOGICALLY INTEREST-ING TRACE ELEMENTS IN THE ABSENCE OF INTERFERING ACTIVITIES (AFTER GUINN AND HOSTE, 1980)

Elements	(n,γ) radioisotope	Half-life $(t_{1/2})$	Gamma ray energy (keV)	Calculated approximate limit of detection (μg)
Mn	^{56}Mn	2.5785 h	846.8	1.3×10^{-6}
V	^{52}V	3.75 min	1434.0	7.4×10^{-5}
Cu	^{64}Cu	12.701 h	511.0	3.5×10^{-5}
As	^{76}As	26.32 h	559.1	2.8×10^{-5}
Hg	^{197}Hg	64.1 h	77.3	1.5×10^{-5}
Cr	^{51}Cr	27.69 d	320.1	7.8×10^{-3}
Ni	^{65}Ni	2.520 h	366.3	7.3×10^{-3}
Mo	^{99}Mo[b]	66.02 h	181.1[b]	6.8×10^{-3}
Cd	^{115}Cd[b]	53.47 h	527.9[b]	6.3×10^{-3}
Mo	^{101}Mo	14.6 min	191.9	3.1×10^{-3}
Cs	^{134}Cs	2.062 y	604.7	2.4×10^{-3}
Hg	^{203}Hg	46.612 d	279.2	2.4×10^{-3}
Zn	^{69m}Zn	13.76 h	438.6	1.2×10^{-3}
Rb	^{86}Rb	18.66 d	1076.6	8.4×10^{-2}
Co	^{60}Co	5.271 y	1173.2	1.2×10^{-2}
Se	^{75}Se	119.770 d	136.0	1.2×10^{-2}
Zn	^{65}Zn	244.0 d	1115.5	4.2×10^{-1}
Fe	^{59}Fe	44.63 d	1098.6	3.2

[a] For $\Phi_{th} = 10^{13}$ n·cm^{-2}·s^{-1}, t_i = 5 h, t_d = 0, t_c = 100 min, Ge(Li) detector of 40 cm^3, sample-to-detector distance of 2 cm, and lergest photopeak. The limit of detection is defined as the amount of the element, in μg, that gives 30 net photopeak counts in the major gamma ray peak of the (n,γ) product of the element that provides the most sensitive detection (Guinn and Hoste, 1980).

[b] In current practice, quantitative measurements are generally based on counting of daughter isotopes, respectively ^{99m}Tc (gamma ray energy: 140.5 keV) and ^{115m}In (gamma ray energy: 336.2 keV) because they are the most suitable radionuclides for counting.

reach the levels of interest in specified biological matrices. In the author's experience, Erdtmann's (1976) neutron activation tables also give useful information. They catalog the activation products in decays per second and per μg of an element after different irradiation times: one second (s), one minute (min), one hour (h), one day (d), 20 days, and at saturation (when S = 1). In general, sensitivities of neutron activation analysis compare very favorably with those obtainable by other techniques.

SOURCES OF ERROR

As with all other techniques, neutron activation is subject to a number of experimental errors. They may be introduced at all stages of the analytical process: during the collection and preparation of the sample, the irradiation, the postirradiation chemical treatment, the radioactive counting or, finally, at the moment of the calculation of the results. In September 1982, the International Atomic Energy Agency convened an advisory group meeting around the issue — its report was published in 1984 (Advisory Group of the International Atomic Energy Agency, (1984)).

In fact, errors at the sampling stage can be more easily avoided with neutron activation analysis than with any other analytical technique: firstly, because preirradiation sample preparation can generally be kept to a minimum and, secondly, because mineralization of the sample and chemical separations of the elements of interest can be postponed till after the end of the irradiation when extraneous additions from reagents and laboratory equipment no longer affect the final result. These points are discussed more in detail later in this chapter.

A common source of error arises when sample and standard are not subjected to the same neutron flux as a cause of horizontal or vertical flux gradients. For accurate analyses, they have to be compensated or eliminated (e.g., by rotating the samples). Self-shielding may also introduce significant errors, namely when there is a difference in absorption by the matrices of sample and standard. In general, biological matrices are poor absorbers of neutrons; however, the phenomenon must be kept in mind when designing the standard.

Among the documented sources of error are interference or threshold reactions. They should be considered when determining an element of atomic number Z in the presence of a large excess of an element of atomic number Z + 1 or Z + 2. Examples of great practical importance for the biomedical researcher are mentioned earlier in this chapter. The resulting errors may be easily calculated according to a formula which may be found in all textbooks on neutron activation analysis. When packed blood cells with an iron content of approximately 1.025 g/kg wet weight are irradiated in a neutron flux with a thermal-to-fast ratio (Φ_{th}/Φ_f) of 10, the $^{56}Fe(n,p)^{56}Mn$ interference reaction gives an "apparent" manganese concentration of approximately 7.6 ng/g, the $^{54}Fe(n,\alpha)^{51}Cr$ reaction an "apparent" chromium concentration of approximately 5.9 ng/g; when serum with a phosphorus content of approximately 135 mg/L is irradiated under the same conditions, the $^{31}P(n,\alpha)^{28}Al$ reaction gives an "apparent" aluminum concentration of approximately 97 ng/ml!

Still other problems can arise when a radionuclide has not been equilibrated with its carrier, when a radiochemical separation is carried out imperfectly, or when the counting efficiency differs for sample and standard (different shapes or sizes, different gamma ray attenuation).

Spectral interferences are mutual interferences of radionuclides emitting gamma rays with similar energies so that they cannot be resolved. In seeking out possible spectral interferences, it is necessary to have a catalog of gamma lines of the radionuclides

arranged according to their energy and a table of radionuclides showing all gamma rays associated with their radioactive decay as in one of the earliest examples published by Adams and Dams (1969). Unfortunately, no catalog explicitly gives all the necessary information even though it is complete and even though the energies included are accurate: e.g., pile-up peaks (peak appearing from the coincident counting of two gamma rays or of an X-ray and a gamma ray) and single or double escape peaks (SE or DE peaks) of high-energy gamma rays (e.g., 1732.0 keV — DE peak of the 2754.0 keV photopeak of ^{24}Na) are practically never listed. When overlooked, some phenomena may introduce very serious errors, e.g., the determination of cobalt via its ^{60}Co radioisotope (gamma rays of 1173.2 and 1332.5 keV) may give highly erroneous results in the presence of a high activity of ^{82}Br (gamma rays of 554.3 and 619.0 keV and 554.3 and 776.5 keV give sum peaks of, respectively, 1173.3 keV and 1330.8 keV)!

ADVANTAGES

Probably the most praised advantage of neutron activation analysis is its relative freedom from errors due to contamination of the sample. Of course, during collection, transport, and subsampling, the same amount of care is needed as for all other techniques for trace elemental analysis (judiciously selected instruments, high-purity collection vessels cleaned with meticulous care, clean room conditions, and so forth). Some problems are even specific for neutron activation analysis, namely irradiation and wet ashing blanks (Cornelis et al., 1982 and Maziere et al., 1976). On the other hand, preirradiation sample preparation can generally be kept to a minimum. With a few exceptions, e.g., if an element can only be determined via a very short-lived radioisotope as in the case of vanadium (Cornelis et al., 1981), mineralization and chemical separations can be postponed until after the irradiation when extraneous additions (impurities in the reagents extraneous additions from laboratory equipment, or airborne contaminations) no longer affect the final result. In the author's opinion, this is one of the most important reasons why the technique contributed to such a large extent to the definitive establishment of reference values for the most difficult trace elements in a wide variety of biological matrices.

There is also a broad measure of agreement that neutron activation analysis is one of the most sensitive (down to the 10^{-6}, 10^{-9} and even 10^{-12} level for certain elements) and specific techniques for elemental analysis. Up to the first half of the 1960s, no analytical techniques could even match the sensitivity of neutron activation analysis. Although since then several other techniques were developed with sufficient sensitivity to permit determination of many trace elements in biological matrices, neutron activation analysis has only infrequently been discarded.

A much publicized advantage is also that the technique is ideally suited for multielement determinations. On the other hand, when looked at objectively this characteristic would appear to have been seriously overestimated by some researchers. In certain papers (Ward and Pim, 1984; Ward and Ryan, 1979), the impression is created that

numerous elements can easily be measured in difficult matrices (e.g., human blood plasma or serum) using relatively simple experimental conditions. A critical examination, however, shows that the vast majority of the data must have been subject to severe analytical errors: indeed, most are grossly overestimated so that no valid conclusions can be drawn! According to the experience of the author of this chapter, the determination of true plasma or serum trace element levels is a matter of great difficulty, requiring stringent experimental conditions. Conclusions based on analyses of commercially available serum samples with unrealistically high levels of most elements (Nadkarni and Morrison, 1976) are also meaningless.

An interesting aspect of instrumental neutron activation analysis is its nondestructive character which is unique among all analytical techniques: after measurements are completed, the same sample can be used for the determination of additional elements using other techniques.

Other virtues of neutron activation analysis are that the relation between the concentration of an element and the measured signal are nearly matrix independent and that radiochemical separations can be carried out in optimal conditions by post-irradiation carrier addition.

An interesting feature is also that all sources of random variation can be identified; it is thus possible to predict the standard deviation of an analytical result: it has been defined as the "a priori precision". In the "analysis of precision" (Heydorn, 1984 and Heydorn and Nørgård, 1973) it is compared with the actually observed variation. The results may serve as a method of continuous quality assessment (Heydorn, 1976).

The preceding considerations will have made it clear that neutron activation analysis may be classified as a powerful analytical technique.

DISADVANTAGES AND LIMITATIONS

In general, neutron activation analysis is expensive when compared to other techniques. Capital costs mentioned in the literature vary from somewhere between US $ 300 000 and US $ 1 000 000 for a low- (Guinn and Hoste, 1980) up to US $ 5 000 000 for a high-flux research type nuclear reactor (Bowen, 1981); in addition, the latter can have total annual running costs of US $ 500 000 (Bowen, 1981) up to US $ 1 000 000 (Been and Riste, 1984). To reduce the expenses, a nuclear reactor is very often shared by various departments in a university or various institutions and industries in a large area.

Modern counting equipment is also rather highly priced: a system with a high-resolution Ge(Li) or HP-Ge detector and a 4000 channel analyzer costs from US $ 45 000 to US $ 60 000 depending on the detection efficiency and resolution of the detector and the data reduction capabilities of the multichannel analyzer. A sample changer to count automatically and computer for data reduction push up the expenses further.

In the past this aspect did not prove to be a deterrent to the extensive application of the neutron activation analysis: straws in the wind indicate that it will assume increasing importance in the future. Certainly, powerful alternative techniques have been developed

and continuously refined; on the other hand, there is no doubt that further curtailing of established neutron activation research facilities would create a serious flaw in trace metal analysis of biological samples.

In a radioanalytical laboratory, a number of safety regulations must be strictly observed. The first step should always be to make a calculation of the expected activity: it is very important because it determines the type of laboratory needed and governs the safety measures and the waste disposal. It should always be considered whether the conditions can be changed in order to reduce the radioactivity or whether a short-lived radionuclide can be used instead of one with a longer half-life.

Most biological matrices contain considerable amounts of sodium and chlorine which, after activation, emit gamma rays with great penetrating power: 1368.6 and 2754.0 keV (^{24}Na) or 1642.4 and 2167.5 keV (^{38}Cl). Thus, 1 mL of serum contains approximately 3.25 mg of sodium and 3.95 mg of chlorine. After irradiation for 5 h in a neutron flux of $4 \cdot 10^{12}$ $n \cdot cm^{-2} \cdot s^{-1}$, the first gives rise to approximately 1 mCi or 37 MBq of ^{24}Na (half-life: 14.959 h), the second to approximately 0.75 mCi or 28 MBq of ^{38}Cl (half-life: 37.21 min). Obviously, ^{24}Na forms the most serious problem.

The preceding will have made it clear that the opening of an irradiation can with biological samples shortly after the end of an irradiation must be done under careful monitoring to avoid unacceptable exposure of the investigator's eyes and hands and that good ventilation is required to prevent the inhalation of radioactive dusts or gases. Furthermore, as long as the high matrix activities are not eliminated, manipulations will preferentially be carried out behind a wall of lead (or lead-glass) but this does not cause a serious problem: in addition, it is evident that several steps can readily be automated – particularly when large numbers of samples of similar nature are to be processed under identical conditions, the devising and building of an automated separation system (Lievens et al., 1977; Tjioe et al., 1973) is worth serious thought.

A detailed discussion of the rules for working in radiochemical laboratories and of radiation protection measures falls outside the scope of this chapter: a good summary and references for further reading are given elsewhere (Lieser, 1986). When experiments are judiciously planned, hazards of handling reactor- irradiated biological samples are seldom more important than those encountered in other work involving the use of radio-nuclides.

An additional hindrance to the use of neutron activation analysis – especially in case of clinical applications – is the long delay between the collection of a sample and the final calculation of the result. Indeed, in extreme analysis – more particularly when long-lived radioisotopes are involved – irradiation periods of several days, even up to a couple of weeks, and measuring periods of tens of hours per sample are often required to attain the desired radioactivity. During the irradiation, matrix elements or major components become intensely radioactive so that long cooling periods – weeks! – may be necessary to allow undesirable isotopes to decay. Fortunately, for some elements, there are several alternatives: e.g., selenium can not only be quantified via its ^{75}Se ($t_{1/2}$ = 119.770 d) (Versieck et al., 1977) but also via its ^{81m}Se ($t_{1/2}$ = 57.3 min) (Heydorn and Damsgaard, 1973) or, even, via its ^{77m}Se radioisotope ($t_{1/2}$ = 17.5 s) (Dickson and Tomlinson, 1967; Hahn et

al., 1972). In summary, however, it may be said that neutron activation analysis is a rather expensive, time-consuming and labor-intensive analytical technique.

BIOMEDICAL APPLICATIONS

Neutron activation analysis has been used by numerous investigators all over the world to measure trace element levels in a wide variety of biological matrices: the references listed by Bowen (1981), Heydorn (1984), Iyengar et al. (1978), Versieck (1985), Versieck and Cornelis (1980) and Versieck and Cornelis (1988) give a very good overview of what has been published. Unfortunately, in a number of publications quality assurance left much to be desired so that the included data should be approached with extreme caution. Earlier in this chapter, reference was already made to the publications of Ward and Pim (1984) and Ward and Ryan (1979). There is no doubt that also in several other studies serious errors must have been made as exemplified by the papers of Astrug et al., 1984; Nakahara et al., 1979; and Nakahara et al., 1980. Table 2 shows a comparison of the figures obtained by these researchers in human blood plasma or serum with the results obtained at the University of Ghent by the author of this chapter in collaboration with his colleagues and co-workers. The severe inconsistencies between the published data illustrate better than an extensive discussion the long distance that remains to be covered before a consensus will be attained. Hopefully, the advent of second-generation biological reference materials with trace element levels close to those in real human blood plasma or serum samples (Veillon et al., 1985 and Versieck et al., 1988) will prove to be an invaluable expedient in this delicate process. The figures in the table show that neutron activation analysis, in spite of its virtues outlined above, is certainly not a panacea. It has been said that the bias of trace element determinations depends more on the analyst than on his technique and what precedes furnishes further support for this thesis. The results published with a one-year-interval by Nakahara and associates are listed intentionally alongside to illustrate how contradictory results obtained in one and the same laboratory may be. Apparently, however, the discrepancies were not a cause of great concern for the authors who state that it can be said that the data for normal men are in reasonable agreement except for the values for cobalt. The dissonant values for chromium are not quoted explicitly.

At the University of Ghent Institute for Nuclear Sciences, neutron activation analysis was used for numerous additional studies in the biomedical field in close co-operation with a number of services at the University Hospital. Most elements were not only studied extracellularly in serum but also intracellularly in packed blood cells (Cornelis et al., 1979; Cornelis et al., 1981; Versieck et al., 1973; Versieck et al., 1974; Versieck et al., 1977; Versieck and Vanballenberghe, 1985; and Wallaeys et al., 1986). The urinary excretion of trace elements was also studied in a few subjects (Cornelis et al., 1975); determinations in liver tissue were done by Lievens and associates (Lievens et al., 1977), in lung tissue by Vanoeteren and colleagues (Vanoeteren et al., 1982; Vanoeteren et al., 1983; and Vanoeteren et al., 1986). Patients with internal diseases were also intensively studied, e.g.,

patients with heart (Versieck et al., 1986 and Versieck et al., 1975), liver (Versieck et al., 1974; Versieck et al., 1976; and Versieck et al., 1981), kidney (Cornelis et al., 1979; Cornelis et al., 1980; Wallaeys et al., 1985; and Wallaeys et al., 1986), and metabolic diseases (Lombeck et al., 1986).

Neutron activation analysis has also been employed sporadically for the determination of trace elements in protein fractions, collagen, nucleic acids as well as in biochemicals containing activatable elements (e.g., cobalt in vitamin B_{12} or selenium in selenoaminoacids).

In clinical practice, most trace element determinations are not done by neutron activation analysis but by other techniques, in the first place atomic absorption spectrometry — a technique having a good sensitivity for numerous elements and better suited for routine application. Nevertheless, neutron activation analysis played an important role as illustrated by the observation that somewhat more than 50% of the selected reference values advanced by Versieck in his 1985 CRC Critical Review were obtained by this technique.

One final important application may be added: the role of neutron activation analysis in the certification of biological reference materials. The experience with Bowen's kale powder was reviewed by the investigator in 1975 (Bowen, 1975), the contribution to the NBS Standard Reference Materials (SRM's) program was commented recently by Greenberg (1987). In the case of the second-generation biological reference material issued by Versieck and colleagues (Versieck et al., 1988), approximately 45% of the results used for certification were obtained by neutron activation analysis.

SUMMARY AND CONCLUSIONS

Neutron activation analysis is an invaluable technique for trace element determinations in biological matrices. Probably its most important advantage is its relative freedom from errors due to extraneous additions of exogenous material from reagents, equipment, or laboratory environment. Characteristics which contribute further to the popularity of the technique are its outstanding sensitivity, excellent specificity, and multielement capability. In principle, the technique is able to produce relatively unbiased and precise measurements — at least in competent hands. That it is, however, necessary to warn against uncritical expectations is illustrated by the grossly inconsistent results obtained in several laboratories.

Because of the necessity to have access to a nuclear reactor, the use of neutron activation analysis has been restricted to a few — essentially research — laboratories, the more so because most analyses require considerable time and manpower. The technique has found its main application in solving selected problems or in laying the foundations for more extensive applications using procedures better suited for routine applications.

To close: neutron activation analysis has had a profound influence on trace element research, particularly on its biomedical applications. Without the development of the technique the history of trace element research would, undoubtedly, have to be rewritten. In

TABLE 2

COMPARISON OF TRACE ELEMENT CONCENTRATIONS (MEANS ± S.D.) DETERMINED IN PLASMA OR SERUM OF APPARENTLY HEALTHY INDIVIDUALS BY DIFFERENT RESEARCHERS USING NEUTRON ACTIVATION ANALYSIS

Element (unit)	Astrug et al. (1984)	Nakahara et al. (1979)	Nakahara et al. (1980)	Ward and Pim (1984)	University of Ghent (different references)[a]
Aluminum (μg/L)		4830 ± 2760	2410 ± 3220	194 ± 25	3.72 ± 1.20[b]
Scandium (μg/L)		0.460 ± 0.036	0.168 ± 0.201	2.4 ± 0.5	–[c]
Vanadium (μg/L)				9.5 ± 1.3	0.024 – 0.939 (♂)[d] 0.031 ± 0.010 (♀)
Chromium (μg/L)	13 ± 11	782 ± 495	2.14 ± 11.4	6.1 ± 1.0	0.160 ± 0.083
Manganese (μg/L)		14.2 ± 26.2	3.49 ± 2.10	2.2 ± 0.4	0.57 ± 0.13
Cobalt (μg/L)	1.9 ± 0.6	89.3 ± 71.3	5.01 ± 3.09	0.37 ± 0.06	0.108 ± 0.060
Copper (mg/L)		2.60 ± 3.57	1.01 ± 0.38	1.01 ± 0.07	1.07 ± 0.24
Zinc (mg/L)	1.38 ± 0.43	3.40 ± 3.88	1.13 ± 0.28	1.13 ± 0.09	0.94 ± 0.13
Arsenic (μg/L)	16 ± 7	3.05 ± 2.26	3.50 ± 1.95	15 ± 4	0.088 ± 5.488[d]
Selenium (mg/L)	0.027 ± 0.007	0.340 ± 0.135	0.196 ± 0.071	0.115 ± 0.012	0.13 ± 0.02
Bromine (mg/L)	2.420 ± 0.120	9.92 ± 4.51	6.0 ± 2.3	3.66 ± 0.20	4.87 ± 2.02
Rubidium (mg/L)	0.540 ± 0.350	0.291 ± 0.339	0.153 ± 0.038	0.111 ± 0.014	0.17 ± 0.04
Molybdenum (μg/L)				2.4 ± 0.4	0.58 ± 0.21
Silver (μg/L)				2.0 ± 0.4	–[c]
Cadmium (μg/L)				2.1 ± 0.4	< 0.192[d]
Tin (μg/L)				34 ± 6	–[c]
Antimony (μg/L)	6.5 ± 1.5	13.4 ± 5.6	3.58 ± 4.16	2.5 ± 0.6	–[c]
Cesium (μg/L)	3 ± 2	0.935 ± 0.075	2.71 ± 0.82	3.0 ± 0.6	0.74 ± 0.20
Mercury (μg/L)				12 ± 3	1.02 ± 0.43[e]

[a] References include Cornelis et al., 1981; Cornelis et al., unpublished results; de Baets et al., unpublished results; Versieck et al., 1973; Versieck et al., 1974; Versieck et al., 1977; Versieck et al., 1978a; Versieck et al., 1978b; Versieck et al., 1985; Versieck et al., 1990.

[b] Results obtained by atomic absorption spectrometry.

[c] As yet no values are available for apparently healthy individuals. Measurements in a second-generation biological reference material (lyophilized human serum) with trace element levels close to those in real human blood plasma or serum (Versieck et al., 1988) yield the following results - for scandium: $0.0017\ \mu g/L$ (Xilei et al., 1988), for silver: $0.085\ \mu g/L$ (Xilei et al., 1988), for tin: $0.91\ \mu g/L$ (Xilei et al., 1988) and $0.78\ \mu g/L$ (Versieck et al., 1991), and for antimony: $0.023\ \mu g/L$ (Xilei et al., 1988) and $0.016\ \mu g/L$ (Byrne, personal communication) (recalculated values $-$ published figures are in ng/g dry or lyophilized weight).

[d] Range or, in the case of cadmium, upper limit.

[e] Preliminary results in a small series of individuals (9 patients).

these days of economic recession, there might be a tendency to reduce the support of established neutron activation analysis research groups: inevitably this would entail a serious drag on the advancement of the correct appreciation of the role played by trace elements in human health and disease.

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Chapter 8

Isotope dilution mass spectrometry (IDMS)

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Quantitative determination by stable[*] isotope dilution is a method which has been known for a number of years [Hintenberger, 1955] [Webster, 1958, 1960] [Riepe, 1966; De Bièvre, 1971] [Heumann, 1988; De Bièvre, 1990]. Three factors have prompted its development:

1. the need for unbiased determinations of (very small) amounts of isotopes in geo-chronology for the establishment of the actual ratio of a parent isotope to its daughter isotope in order to determine ages
2. the need for unbiased assay of fissionable materials in complex radioactive solutions
3. the availability of enriched stable isotopes of most of the elements from the electro-magnetic separators in Oak Ridge, Tennessee (U.S.A.).

The method requires mass spectrometric measurements of ratios of stable or long lived isotopes and can be applied to about 75 elements, from which about 50 can be measured as solid compounds by thermal ionization mass spectrometry.

IDMS is best illustrated by its most simple case: the determination of an unknown number N_X of atoms of a monoisotopic[*] element, say ^{127}I. When approximately a same and known number N_Y of atoms of a sufficiently stable isotope ^{129}I of that element is added to this sample, a homogeneous mixture or "blend" of both isotopes can be made and the isotope ratio $R = {}^{127}I/{}^{129}I$ measured in that blend as R_B (see Fig. 1a). Then:

$$\frac{N_X}{N_Y} = R_B \qquad (1)$$

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[*] "nuclide" is the correct term, so is "nuclidic", "mononuclidic", "polynuclidic" etc.; however, "isotope", "isotopic", "monoisotopic" etc. have become common parlance for so many years that we will continue to use them.

The known amount N_Y added is called a "spike". R_X and R_Y can be defined as the isotope ratios R of the unknown sample X and of the "spike" respectively. Note that in this example — since the unknown sample is monoisotopic (containing ^{127}I only) — $R_X = \infty$ and that $R_Y = 0$ if a pure ^{129}I isotope is used as spike material. In fact an isotope is seldom, if ever, 100% pure. Consequently it is better to use the detection limit for the "non-existent" isotope, With such a limit, at, say 1 atom in 10^6 atoms, $R_X = 10^6$ and $R_Y = 10^{-6}$.

Since a mass spectrometer can also measure ratios R_B much smaller or larger than 1, the "spiking" can be performed over wide ranges of the sample to spike ratio N_X/N_Y (see Figs. 1b, 1c).

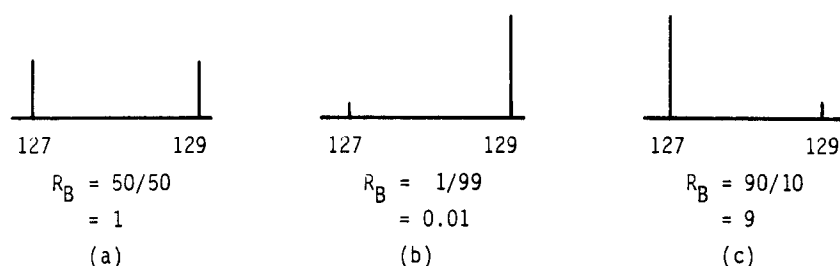| 127 129	127 129	127 129
R_B = 50/50	R_B = 1/99	R_B = 90/10
= 1	= 0.01	= 9
(a)	(b)	(c)

Fig. 1. Various sample to spike ratios are possible in IDMS.

From this simple example the basic advantages and requirements of quantitative determinations by isotope dilution can be deduced.

Advantages:
1. *precise (= reproducible): isotope ratios can be measured very precisely ($\leq 0.2\%$) by isotope mass spectrometry*
2. *unbiased: the determination can be performed to an uncertainty equal to the sum of uncertainties of the isotope ratio measurement in the blend (0.05% - 1%) and of the spike definition (0.01% - 1%)*
3. *the assay of the spike N_Y can be performed on a large amount of pure material (either solid or solution) and only small but well defined fractions (by weighing) of that material are needed in the spiking process; the uncertainty of the spike can hence be small and well known*
4. *sensitive: the sensitivity is only limited to the sample size needed for an isotope ratio measurement of the blend: anywhere from 10^{-4} g to very small amounts (10^{-12} g) depending upon element and instrumentation*
5. *selective: the determination is highly selective because it is only based on the measurement of the ratio of isotopes of the same element, with very few interferences possible (isobaric interferences in the mass spectrum); the latter can easily be*

avoided either by chemical elimination (separation) or correction (via known isotopic composition of the interfering element)

6. sample treatment need not to be quantitative once the spike has been added and mixed homogeneously to the sample thereby fixing the isotope dilution ratio in the blending process: each sample of any size carries the required ratio R_B

7. only a very rough estimate of the unknown sample amount is required to choose spiking ratios; the resulting ratio R_B should only fall into the wide dynamic measurement capability of an isotope mass spectrometer (several orders of magnitude)

8. because of point 7, very small samples (or very small concentrations in a sample), difficult or impossible to handle otherwise, can be spiked with a 100- or 1000-fold larger spike amount, thus increasing the size of the blend to a size which can be handled and measured.

Requirements:

1. a separated isotope other than the one of the sample must be available; however, as we will see further on, same isotopes as in the sample but with other abundances ("isotopically enriched") are also suitable

2. homogeneous mixing of sample and spike; this is absolutely essential but not basically difficult to achieve

3. recovery from the blend of a sample for determination of the isotope dilution ratio, without any alteration of this ratio; this is a problem of avoiding isotopic contamination of samples by reagent blanks, separation columns, glassware, dust, etc.; however, these problems arise only when very small samples (submicrogram) are handled

4. a mass spectrometer to measure isotope ratios either on solid compounds (for about 50 elements) or on gases

5. separation techniques to separate (small) amounts of the isotopic blend from larger samples of materials (solutions or solid matrices)

6. a "spike", assayed or certified to a well known accuracy.

We now proceed to a slightly more complicated example: that of a biisotopic element, say lithium with isotopes ^6Li and ^7Li and an isotope ratio $R = {}^6Li/{}^7Li$:

(a)   7.5 % ^6Li (b)   95 % ^6Li (c)   50 % ^6Li
        92.5 % ^7Li 5 % ^7Li 50 % ^7Li

$R_X \cong 0.08$ $R_Y \cong 19$ $R_B \cong 1$
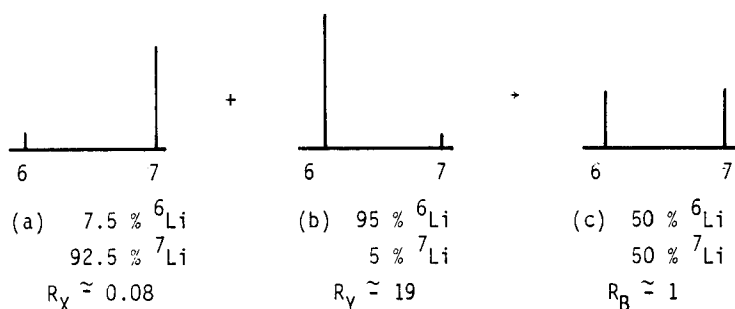
Fig. 2. IDMS of bi-isotopic elements.

P. De Bièvre

We observe

$$\frac{N_X}{N_Y} = \frac{(N_6 + N_7)_X}{(N_6 + N_7)_Y} = \frac{R_X + 1}{R_Y + 1} \cdot \frac{(N_7)_X}{(N_7)_Y} \tag{2}$$

and

$$R_B = \frac{(N_6)_X + (N_6)_Y}{(N_7)_X + (N_7)_Y} = \frac{R_X(N_7)_X + R_Y(N_7)_Y}{(N_7)_X + (N_7)_Y}$$

hence:

$$\frac{(N_7)_X}{(N_7)_Y} = \frac{R_Y - R_B}{R_B - R_X} \tag{3}$$

Combination of equations (2) and (3) yields

$$\frac{N_X}{N_Y} = \frac{R_Y - R_B}{R_B - R_X} \cdot \frac{1 + R_X}{1 + R_Y} \tag{4}$$

It is seen that the ratio of an unknown number of atoms N_X to a known number N_Y of atoms ("spike") is again solely a function of isotope ratios. However, also sample and spike have to be measured for their isotopic composition (measurement of R_X and R_Y) in addition to the blend (measurement of R_B). This can be performed on separate samples of unknown and "spike" material and does not require quantitativity in sample-taking. It is trivial to note that the same reference isotope must be used in sample, spike and blend ratios. However, any isotope can be used as reference isotope.

(a) 5.8 % ^{54}Fe	(b) 98 % ^{54}Fe	(c) 52 % ^{54}Fe
- ^{55}Fe	- ^{55}Fe	- ^{55}Fe
91.7 % ^{56}Fe	1 % ^{56}Fe	46.3 % ^{56}Fe
2.2 % ^{57}Fe	0.5 % ^{57}Fe	1.3 % ^{57}Fe
0.3 % ^{58}Fe	0.5 % ^{58}Fe	0.4 % ^{58}Fe

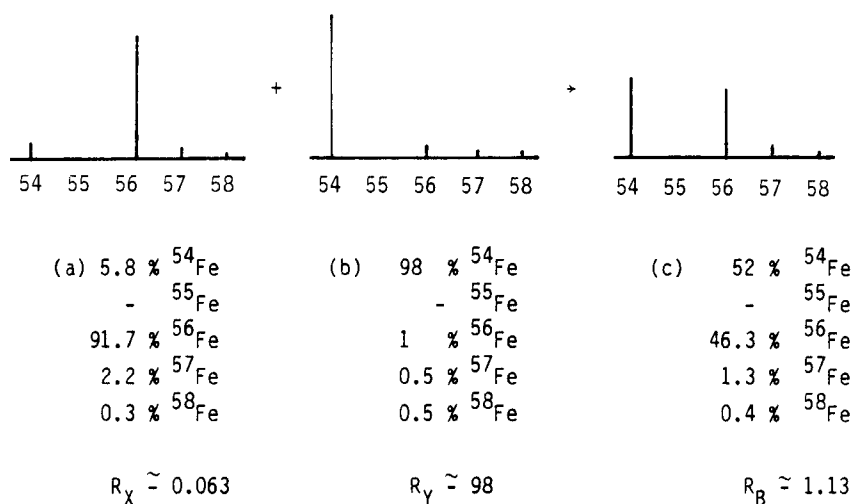$$R_X \simeq 0.063 \qquad R_Y \simeq 98 \qquad R_B \simeq 1.13$$

Fig. 3. IDMS of poly-isotopic elements.

We now take to the most general and complex case of isotope dilution: sample and spike are polyisotopic. An example is that of iron where we choose arbitrarily $R = {}^{54}Fe/{}^{56}Fe$.

By measuring in the blend the **change** in the ${}^{54}Fe/{}^{56}Fe$ ratio caused by adding the "spike" of other isotopic composition to the sample and by measuring the other isotopes to make appropriate corrections, one can determine the unknown amount of N_X of sample. The general isotope dilution equation is:

$$\frac{N_X}{N_Y} = \frac{R_Y - R_B}{R_B - R_X} \cdot \frac{\sum R_{iX}}{\sum R_{iY}} \quad \text{or} \quad \frac{C_X M_X}{C_Y M_Y} = \frac{R_Y - R_B}{R_B - R_X} \cdot \frac{\sum R_{iX}}{\sum R_{iY}} \qquad (5)\ (6)$$

where C is concentration in mole per mass unit and M a mass of material expressed in the same mass units as in C.

$\sum R_i$ is the sum of the ratios of all isotope abundances to one isotope abundance chosen as reference. This equation is derived hereafter:

$$\frac{N_X}{N_Y} = \frac{(N_1 + N_2 + ...)_X}{(N_1 + N_2 + ...)_Y} = \frac{(R_1 + R_2 + ...)_X N_{1X}}{(R_1 + R_2 + ...)_Y N_{1Y}}$$

When N_1, N_2 ... are the number of atoms of each isotope and R_1, R_2 the isotope ratios (reference : isotope N_1).

We note that $R_1 \equiv 1$ and choose isotopes 1 and 2 as the ones (and the only ones) to be measured in the blend:

$$R_B = \frac{N_{2X} + N_{2Y}}{N_{1X} + N_{1Y}} = \frac{R_X N_{1X} + R_Y N_{1Y}}{N_{1X} + N_{1Y}} \quad \text{or} \quad \frac{N_{1X}}{N_{1Y}} = \frac{R_Y - R_B}{R_B - R_X} \qquad (7)\ (8)$$

Equations (7) and (8) yield equation (5).

It is seen that also in the general polyisotopic case the ratio of sample and spike amounts can be determined solely from isotope ratio measurements. **Isotope dilution mass spectrometry measures the change in the ratio of two isotopes of the element of interest, induced by the addition of a known amount of the same element with an artificially altered isotope ratio of the same isotopes to a weighed aliquot of a sample.** Since only isotope ratios in the different materials are measured, it follows that sample treatment or recoveries for chemical separations need not be quantitative once isotopic equilibration after spiking has been achieved. Also variability of chemical separation recovery is generally not important.

Up to now we have implicitly assumed that the unknown sample had natural isotopic composition and that the spike consisted of a non-natural mixture of isotopes.

Performing chemistry with a very low bias to determine the exact amount of spike N_Y would, however, be very difficult and require a large (and hence costly) amount of isotopically enriched material. The solution to this can be another isotope dilution i.e. assaying the spike Y (in solution for example) with accurately assayed material Z of

natural isotopic composition. We call this "reverse isotope dilution". The material Z should then be a highly pure element or chemical compound of that element with known (high) purity and stoichiometry. It must also have the same (usually natural) isotopic composition as the material X or deviate very little from that.

Equations for reverse isotope dilution are very simply derived from the "one-way" isotope dilution case described in Eq. (1):

$$\frac{N_X}{N_Y} = \frac{C_X M_X}{C_Y M_Y} = R_B \quad \text{and} \quad \frac{N_Z}{N_Y'} = \frac{C_Z M_Z}{C_Y M_Y'} = R_B' \tag{9) (10}$$

where from the solution of spike material Y with concentration C_Y, a fraction M_Y with N_Y atoms is taken for the determination of the unknown N_X (or C_X) and a fraction M_Y' with N_Y' atoms for the determination of N_Y' (or C_Y) against material Z.

Combination of equations (9) and (10) yields

$$\frac{C_X M_X}{C_Z M_Z} = \frac{M_Y}{M_Y'} \cdot \frac{R_B}{R_B'} \tag{11}$$

The unknown concentration of C_X can be determined from
1. the known concentration C_Z
2. ratios of masses of solutions used
3. ratios of isotope ratios measured.

For isotope dilution of **bi-isotopic and poly-isotopic elements** the equation is the same:

$$\frac{C_X M_X}{C_Z M_Z} = \frac{M_Y}{M_Y'} \cdot \frac{R_Y - R_B}{R_B - R_X} \cdot \frac{R_Z - R_B'}{R_B' - R_Y} \tag{12}$$

since $\Sigma R_{iX} = \Sigma R_{iZ}$ when Eqs. (5) and (6) are written out twice ("one-way ID of X against Y and the "reverse" ID of Y against Z).
Also in the most general case the unknown concentration C_X is determined from
1. the known concentration C_Z of material of the same isotopic composition
2. ratios of masses of solutions used
3. ratios of isotope ratios measured
Note that Eq. (12) is identical for a bi-isotopic and a poly-isotopic element.

Conclusion:

Spike material has to be an isotopically enriched material, is hence available to limited amounts only and/or can be expensive. Reverse isotope dilution can be used to perform its assay because it can be performed on minute amounts or very small concentrations (μg.ml^{-1}).

Furthermore, complete or precise isotope analysis of the spike material is not essential.

Uncertainty considerations:

Equation (1) makes clear that the uncertainty of an IDMS assay consists of the sum of the relative uncertainty of the "spike" and the relative uncertainty of the measurement of an isotope dilution ratio R_B. This statement is valid for both the uncertainty generated by the imprecision (or reproducibility) of the measurement of "spike" and R_B as well as by the systematic error(s) in the measurement of the "spike" and of the isotope dilution ratio R_B.

We will now see that this is equally valid for IDMS of bi-isotopic elements (Eq. 4 and Fig. 2) and in the poly-isotopic case (Eq. 5 and Fig. 3). We note that R can be chosen 2 ways: the major isotope abundance being in the denominator or in the nominator of R_X or R_Y. We arbitrarily choose the latter, hence $R_Y \gg 1$ and quite different from R_B (≈ 1). We note that R_Y is then little affected by R_B. Also because R_Y appears in both nominator and denominator of Eq. 4, any uncertainty on R_Y, will have very little effect on the uncertainty of N_X/N_Y. On the other hand R_X will be small (the major isotope abundance will now be in the denominator of R_X) with respect to R_B or to 1, so any uncertainty on R_X will not contribute to the uncertainty of N_X/N_Y. Consequently only the uncertainty of R_B in the denominator will largely determine the uncertainty of N_X/N_Y and hence of the IDMS result. **The precision with which an isotope dilution ratio can be measured, determines the precision of an IDMS determination** as far as the isotopic measurement is concerned. Further limitations are, or may be:

a) the precision and accuracy of the spike (N_Y)

b) the blank determination (which is a sort of N'_X) when the latter becomes significant relative to the unknown (N_X).

A very important observation at this point is that an IDMS assay is in principle a physical measurement since it is a measurement of ratio of isotopes and not of a ratio of elements (as in classical analytical chemistry). Indeed: two numbers of atoms are compared in a ratio determination and these atoms belong to the same element. Hence all the chemical interferences, normal in a chemical assay, do not affect the result anymore. Combined with the fact that the requirement of being quantitative — essential and difficult in classical chemistry assay — must not be fulfilled (after spiking), this means that IDMS ranks higher in the hierarchy of methods than normal elemental assay methods since it is far less subject to potential chemical error sources. In other words its inherent potential for good precision and accuracy (i.e. small overall uncertainty) and — at least as important — **the transparency of the uncertainty propagation** in (Eqs. 4 and 5) give it the character of what some have called a "reference method" or even a "definitive method".

Does this mean that there are no systematic errors involved in the measurement of a dilution ratio (and of isotope ratios in general)? Certainly not. Isotopes differ in one property: mass. Therefore, any process sensitive to mass effects will affect their measurement. But

1. these processes are small (<0.01%) in most of the normal chemical reactions

2. the effect has a theoretical limit $\sqrt{M_2/M_1}$, hence the possible error has a theoretical maximum

3. where the effect matters — and that is at the occasion of the isotope ratio measurement in a mass spectrometer — it can be adequately corrected for, either by synthetic isotope mixtures or established Isotopic Reference Materials (IRMs)

4. when applying reverse isotope dilution, the correction for systematic errors (mainly isotope fractionation) and usually represented by the correction factor K to be applied to the measurement of R_B and R'_B, cancels. This is easily seen in Eq. 11 and 12[*].

The result of all this is that IDMS is a process which is well known and understood in each of its stages, that the origins of systematic errors are well known and that these errors can be adequately corrected for. These are about all the qualities one likes to expect from a reliable, unbiased assay with small and probable uncertainties.

CHOICE OF OPTIMAL CONDITIONS FOR ISOTOPE DILUTION BY SELECTING APPROPRIATE SAMPLE TO SPIKE MIXING RATIOS

Spiking of an unknown sample can be performed over a wide range (several orders of magnitude) of values for N_X/N_Y. However, this depends upon the isotopic enrichment of the spike. The more the selected isotope ratio differs in the spike from that of the sample, the greater the change the spike will induce in the same isotope ratio of the sample and the higher the sensitivity of the measurement. On the other hand, the better the isotopic enrichment of the spike, the less spike is needed to induce such a change. In general the problem is one of finding the appropriate sample to spike mixing ratios for the given available spike. It is possible to determine the optimal conditions for sample to spike ratio and spike isotopic composition, in order to arrive at the best precision in the determination. First we observe that the relative standard deviation of an isotope ratio measurement is constant over a large range of isotopic compositions (several orders of magnitude, say, 10^{-2} to 10^{+2}) when measurement procedures and instrumentation are performing correctly, hence

$$\frac{\sigma(R_X)}{R_X} = \frac{\sigma(R_Y)}{R_Y} = \frac{\sigma(R_B)}{R_B} = \varepsilon \tag{13}$$

We now take back to Eq. 4, put $N_X/N_Y = q$, perform partial differentiation with respect to the variables R_X, R_Y and R_B, divide by q and eliminate R_B:

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[*] In Eq. 12 it even cancels on R_X, R_Y and R_Z provided K is independent of R as should be the case in a correct measurement procedure (De Bièvre 1976).

$$\left[\frac{\sigma(q)}{q}\right]^2 = \frac{(1 + q)^2}{q^2(R_Y - R_X)^2(1 + R_Y)^2} \cdot \varepsilon^2 R_Y^2$$

$$+ \frac{(1 + q)^2(1 + R_Y)^2}{(R_Y - R_X)^2(1 + R_X)^2} \cdot \varepsilon^2 R_X^2$$

$$+ \frac{[qR_X(1 + R_Y) + R_Y(1 + R_X)]^2[q(1 + R_Y) + (1 + R_X)]^2}{q^2(R_Y - R_X)^2(1 + R_X)^2(1 + R_Y)^2} \tag{14}$$

The relation expresses the dependence of the relative precision of q from all the parameters of the isotope dilution process. The error magnification factor is defined as the ratio between the relative precision $\sigma(q)/q$ of q to the relative precision of the measurement of an isotope ratio $\sigma(R)/R = \varepsilon$. Or, in other words, the magnification factor is the relative precision of q measured in units ε. From Eq. (14) it appears that the magnification factor is a function of q, R_X and R_Y. In Figs. 1 through 8 its square is graphically presented as a function of q and R_Y for different discrete values of R_X.

The typical R_X values presented here are 0, 0.1/99.9, 0.5/99.5, 1/99, 4/96, 10/90, 20/80, 50/50. The magnification factors indicated by the graphs, allow to compute the uncertainty to be expected on a given quantitative determination of an element by IDMS. The assumption $\sigma(R) = \varepsilon R$ (Eq. (13)) is not applicable to very enriched and depleted samples, and in those cases the uncertainties are worse than indicated by the graphs. However, good mass spectrometers and measurement procedures achieve a constant uncertainty on R measurements for values from 0.01 to 100, i.e. over four useful orders of magnitude. Attention is drawn on the fact that an isotope ratio can be defined as either N_2/N_1 or N_1/N_2 and therefore it is always possible to choose $R_X \leq 1$.

From these graphs optimal "spiking" conditions can be selected with associated error magnification factors to be used in conjunction with the measurement uncertainty on R_B. These optimal conditions do not change considerably when samples and/or spikes are poly-isotopic. The reason for this is again that an isotope dilution measurement involves basically the measurement of a change in the ratio of two isotopes only: one mainly coming from the sample, the other mainly originating from the spike. Other isotopes than the two "dilution isotopes" must be measured but do not change the optimal conditions of the spiking process.

EXAMPLES OF THE USE OF THE GRAPHS

1. A precise quantitative determination of a natural boron sample ($R_X \approx 20/80$) estimated at ≈ 0.1 mmol (≈ 1 mg) is required with a 0.5% relative precision. Available spikes range typically from 90 to 99.9% ^{10}B, i.e. $R_Y = 10$ to 1000 and a boron isotope ratio measurement can be performed with a relative precision ε of 0.2%. Fig. 7 shows different interesting parameter combinations.

(a) R_Y = 10: q = 0.7 yields a minimum magnification factor of $\sqrt{2.1}$. The required 0.5% precision means that the magnification factor should be smaller than 0.5/0.2 or 2.5 (when squared : 6.25).

This is the case for $0.11 \leq q \leq 4.8$ and the spike added should be between 0.6 and 0.033 mmol (or between 6 and 0.33 mg).

(b) R_Y = 100: optimum condition at q = 0.2 and magnification factor 2.5 maintained for $0.01 \leq q \leq 5.5$.

(c) R_Y = 1000: optimum condition at q = 0.1 and magnification factor 2.5 maintained for $0.001 \leq q \leq 5.5$.

This example demonstrates that no substantial reduction of the magnification factor is achieved by the use of a highly enriched ^{10}B spike, but that the magnification factor is just maintained for a wider range of q-values.

2. A very small natural boron sample ($R_X \approx 20/80$) has to be assayed, for example 10^{-10} mol (10^{-9} g). One would like to add as much spike as possible in order to dispose of a sufficient amount of material for isotopic analysis, say 10^{-8} mol (10^{-7} g), therefore q = $N_X/N_Y = 10^{-10}/10^{-8} = 10^{-2}$.

Examination of figure 7 yields:

(a) R_Y = 10: the preferred spike amount of 10^{-8} mol (i.e. q = 10^{-2}) will allow to reach a magnification factor of 300 only and therefore an imprecision of 0.2% · $\sqrt{300}$ = 3.4% only.

(b) R_Y = 100: addition of 10^{-8} mol spike (q = 10^{-2}) will allow to reach a magnification factor of $\sqrt{6.5}$, hence an uncertainty of 0.5%.

(c) R_Y = 1000: in this case 10^{-8} mol spike added results in a magnification factor $\sqrt{1.2}$ or an uncertainty on the determination of 0.2% · $\sqrt{1.2}$ = 0.22%.

REMARKS CONCERNING OPTIMAL ISOTOPE DILUTION WORK

A careful examination of Figs. 1−8 leads to some interesting points with respect to conditions needed in order to reach smallest possible uncertainties (in other words: smallest possible magnification factors).

1. The smallest magnification factor possible is 1. Consequently the uncertainty of an IDMS assay is at best equal to the uncertainty ε of the measurement of an isotope ration (R_B) added up to the uncertainty of the spike.

2. To attain smallest uncertainty on a quantitative determination by IDMS, a proper choice of sample to spike ratio has to be made. This implies the estimation a priori of the unknown sample amount. This estimation is **not critical** when the proper spike (R_Y) is available. For instance, (see Fig. 4) for an unknown amount of an element with isotope ratio R_X = 1/99 a precision ε $\sqrt{5}$ is obtained with $R_Y \geq 10$ for $0.09 \leq q \leq 90$. (Note: if the estimated q value is experimentally found outside these limits, then the assay can be done in two steps, using the first determination in order to obtain a first estimate. It is obvious however to look for this first approximation by another less precise method.)

3. The lack of a suitable spike (R_Y) can be more prohibitive for good measurements than the measurement imprecision inherent to the techniques. Fig. 8 shows that for $R_X = 1$ a precision of $\varepsilon \sqrt{5}$ is only obtainable for $0.022 \leq q \leq 1.5$ with $R_Y = 10^{-2}$ or 10^2 i.e. the spike should be highly enriched in one of the two dilution isotopes. However, a spike with $R_Y = 0.1$ or $R_Y = 10$ would yield the same result if a q value of 0.6 ± 0.1 is used.

4. Very enriched or depleted spike material is not always needed; generally a variety of parameter combinations allows to reach an uncertainty close to the theoretical optimum which is ε for the given ratio measurement technique.

5. The uncertainty of an IDMS assay decreases when the difference between R_Y and R_X decreases and the uncertainty is infinite for $R_Y = R_X$ (see Fig. 6 for $R_X = 10/90$ and $R_Y = 10^{-1} = 10/100$); however very small differences between R_Y and R_X still allow a rough determination (in the example R_X and R_Y are not exactly identical, so a measurement can still be done).

6. For very small sample sizes on which direct isotopic measurement cannot be made, it is still possible to choose $q \leq 10^{-2}$ in order to reach a sufficient amount for analysis. In this case the isotopic composition of the sample must be known approximately without measurement (the sample is too small). For "natural" samples this condition is in fact fulfilled. For example: a 10^{-3} mol natural U sample ($R_X = {}^{235}U/{}^{238}U = 0.007$ – see Figs. 3 or 4) can be "spiked" with a hundredfold amount ($q = 10^{-2}$) of 99% enriched ^{235}U ($R_Y = 99/1 = 10^2$). The measurement can be done with and uncertainty of approximately $\varepsilon \sqrt{5}$.

Applications of IDMS on trace elements in biological materials

So far IDMS has been used in a number of assays of trace elements in biological materials. Some of the published cases are:

Ca in serum [Cali et al. 1972] [Moore et al. 1972]

K in serum [Velapoldi et al. 1979] [Gramlich et al. 1982]

Chloride in serum [Velapoldi et al. 1979]

U in biological tissues [Kelly et al. 1983]

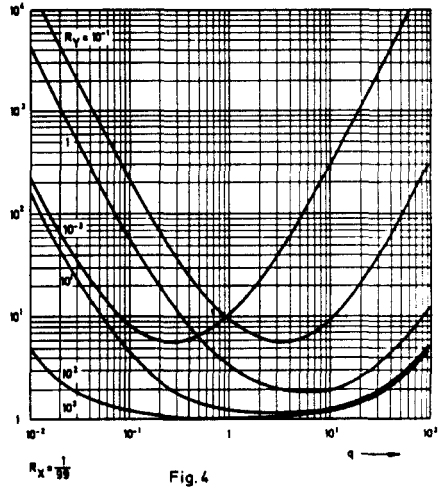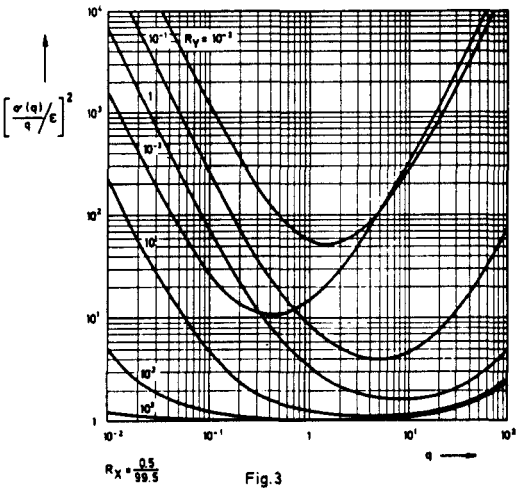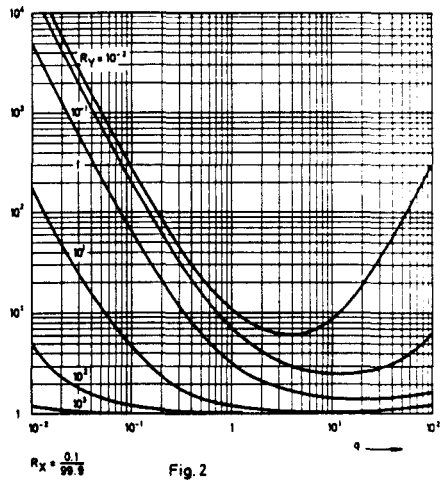Li in serum [Velapoldi et al. 1980] [Michiels et al. 1983]

Pb in various biological materials [Everson et al. 1980] [Trincherini et al. 1983] [Heumann et al. 1981] [Barnes et al. 1982]

Cd in blood [Michiels et al. 1985/6]

Se in biological materials [Raemer et al. 1981]

Tl in biological materials [Heumann et al. 1981]

B in rye grass BCR 281 [Lamberty et al. 1988]

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig.5 $\left[\frac{\sigma(q)}{q}/\varepsilon\right]^2$ vs q, $R_X = \frac{4}{96}$, curves labelled $R_Y = 10^{-1}$, 10^{-2}, 1, 10^1, 10^{-3}, 10^2, 10^3

Fig.6 $R_X = \frac{10}{90}$, curves labelled 10^{-1}, $R_Y = 1$, 10^{-1}, 10^1, 10^{-3}, 10^2

Fig.7 $R_X = \frac{20}{80}$, curves labelled 10^{-1}, $R_Y = 1$, 10^1, 10^{-2}, 10^{-1}, 10^3

Fig.8 $R_X = \frac{50}{50}$, curves labelled $R_Y = 10^{-1}$, 10^1, 10^{-2}, 10^{-3}, 10^3
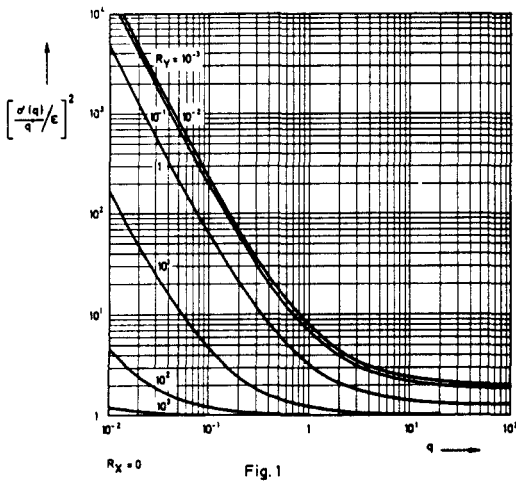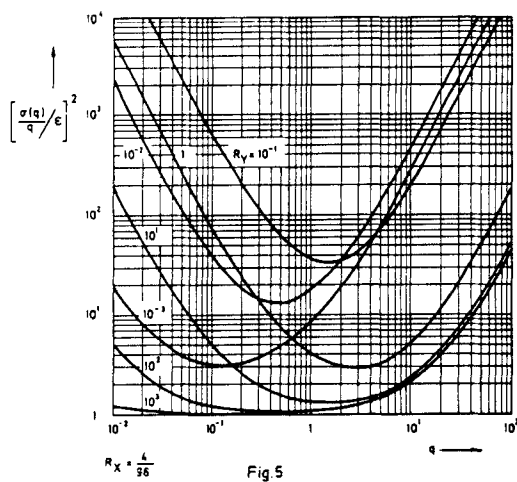
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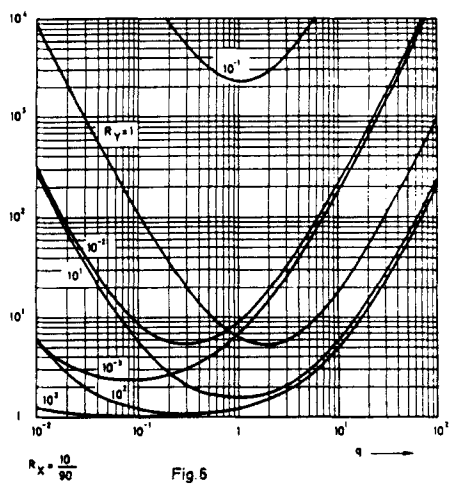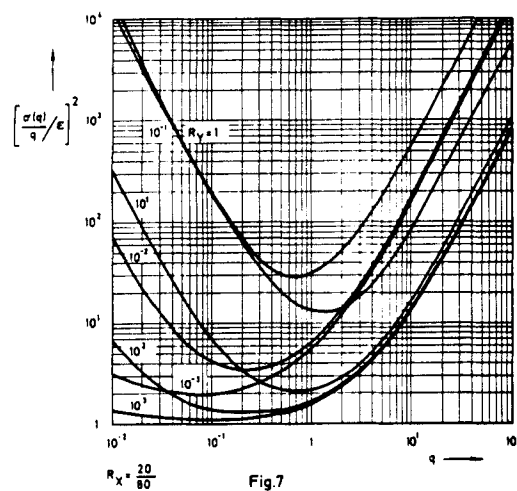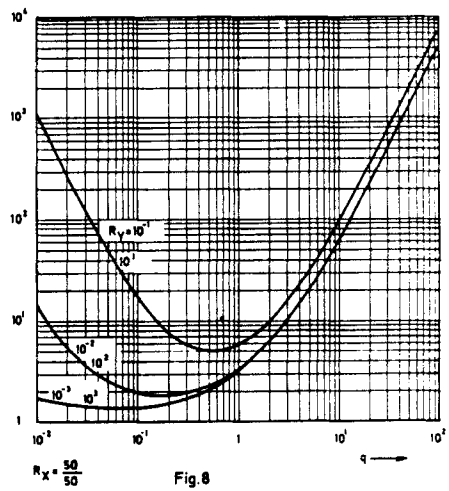Barnes, I.L., Murphy, T.J., Michiels, E. (1982). Certification of lead concentration in standard reference materials by isotope dilution mass spectrometry, J. Assoc. Off. Anal. Chem. 65, 953-956.

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Chapter 9

The chemical speciation of trace elements in biomedical specimens: Analytical techniques

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INTRODUCTION

The rates and extents of trace element dependent metabolic reactions are controlled by the activities of the relevant element-containing species. Determinations of total trace element concentrations in a compartment are of limited value because these represent the sum of all species involved in storage and transport of the trace element in addition to the physiologically active species. It is necessary to identify and quantify all of these species in order to understand at the molecular level the roles of these elements in human metabolism. Areas of clinical chemistry which would benefit from such investigations are:
(i)   deficiencies of essential trace elements; identification of the physiologically most active forms of the trace element-containing species would lead to more effective treatments of deficiency states.
(ii) toxicity of trace elements; identification of the storage and transport species for absorbed trace elements would provide information leading to the design of selective chelating agents used to treat metal overload.
(iii) therapeutic trace elements; studies of the fate of element-containing drugs used in chemotherapy could lead to a better understanding of the biochemical activities of the drugs and thus to improved methods of drug design and of drug administration.
The techniques used for the study of the various physico-chemical forms of a metal or metalloid have to fulfill two major requirements. First, the metalloid- and metal-ligand interactions should not be disrupted before and during the fractionation step, so that the results obtained after separation should reflect as accurately as possible the true distribu-

tion of the species *in vivo*. Second, the detection techniques should have adequate sensitivity and selectivity. This is important as the total concentration of analyte falls below 1 mg/L at which level it is difficult to determine the metal or ligand contents of species, that account for only a few percent of the total.

Over the last decade, developments in both instrumentation and techniques have made it possible to fractionate and determine less than nanogram quantities of various analytes in biological matrices. In this chapter a survey of the available techniques will be given, and the major considerations in the choice of techniques applied to the study of the various chemical forms of an element will be discussed.

FACTORS THAT AFFECT THE STABILITY OF METAL-LIGAND INTERACTIONS

Trace metals interact with proteins, nucleic acids, amino acids, lipids and other ligands to form complexes of varying degrees of thermodynamic stability and reactivity. In order to maintain the integrity of the metal-ligand association the experimental conditions should be such that the complexes remain unchanged both during sample collection, storage and pre-treatment.

On taking a sample from the organism, changes occur in pH, ionic charge, ionic composition, temperature, and enzyme activity. Furthermore in response to this trauma a number of biochemical reactions are triggered.

For example, *in vivo*, the pH of human serum lies in the range 7.35-7.45, and has a temperature of about 37°C. After sampling the pH gradually increases to values over 8.0 depending on the length and method of storage. In addition, the sample attains the temperature of its new surroundings in a very short time. Both factors, as will be shown later, affect the stability of complexes. Proteolysis during storage may cause the metal under investigation to become associated with only a fragment of the ligand(s). Other changes that may occur include denaturation of the proteins and the inhibition of enzymatic activity. As a consequence of the above changes, biochemical reactions that continue *in vitro* may also introduce new chemical species.

In order to reduce the effect of some of these changes, it is essential that samples are either processed soon after collection or stored at temperatures below −20°C. A temperature of −130°C may be required in order to maintain the integrity of cell membranes. However, it has recently been shown that some urinary enzymes rapidly lose activity when stored at low temperatures (Matteucci et al., 1991). It is therefore essential when studying the chemical forms of an element in a given sample to examine the effect of storage on the distribution of the chemical species.

According to Vallee and Coleman (1964) the association between metals and proteins can be divided into two groups namely metalloproteins and metal-protein complexes. In metalloproteins the metal forms an integral part of the protein structure, and it is present in stoichiometric amounts. Furthermore, the metal is not lost during the purification step. The transition metals have a tendency to form this type of association. These complexes have high thermodynamic stability and are relatively inert. On the other hand, in metal-protein

complexes the metal is tenuously bound, and it could be lost during the purification step. Although these complexes may be thermodynamically stable, they are in most cases labile. Metals that form this type of association are mainly the alkali, alkaline earth and other main group elements. The tendency to form inert complexes increases with charge. Although the above classification involves proteins, it can also be applied to other biological ligands.

Both the thermodynamic stability and the reactivity of a complex are influenced by the changes in experimental conditions such as pH, ionic strength, temperature, and redox potential.

A number of studies on the thermodynamic stability and reactivity of biological complexes of trace metals have been reviewed (Vallee and Wacker, 1970). More recently, a book edited by Burger (1990) deals with aspects of this topic. It is difficult to predict with any certainty the behaviour of these complexes during fractionation. However, the changes in experimental conditions that affect the stability of known inorganic and organic complexes introduced into an aqueous medium are instructive (see for example, Beck 1970), and some of the findings will be used here. In addition, experimental parameters that affect the distribution of the species will be discussed.

Further discussion on the behaviour of interacting molecules during fractionation can be found in a work by Cann (1970).

The Effect of pH on the Trace Metal Distribution

The pH value of biological systems is maintained within a narrow range, deviation from which could result in the displacement of the metal from the ligand, inhibition of enzyme activity, precipitation of some species and denaturation of proteins. The rate of substitution of ligands and metals in labile complexes may be altered resulting in a shift in the equilibrium of various reactions. In order to minimise these changes it is essential to buffer the pH during fractionation to near or within the physiological range. A list of the pH of various human fluids and materials can be found in the Handbook of Chemistry and Physics.

The buffer used in the fractionation of trace metal bound species should meet five criteria:

(i) It should not bind the metal, ligand or separation matrix. A number of buffers contain ligands that can effectively compete with the proteins for the trace metals and so alter the distribution. When data on complex formation between ligands and various metals under physiological conditions are not readily available, two or more buffers with different chemical structures should be tried, and if consistent results are obtained the absence of interaction between the metals and ligands in the buffer may be assumed.

A list of commonly used biochemical buffers and their interaction with some metal ions have been summarised by Good and Izawa (1972).

(ii) The buffering capacity must be good in the required pH range particularly when one is dealing with labile complexes because small changes in pH may lead to the displacement of the metal or ligand by hydrogen or hydroxyl ions.

(iii) The buffer should be available in a high purity form. A number of biological ligands can bind trace metals *in vitro*; therefore it is essential to ensure that these metals are not introduced into the sample from an external source during the analytical procedure. As a rule of thumb, the trace metal contamination should be less than 10% of the total concentration of the metal in the sample. Besides the contribution from the metals, other impurities introduced into the buffer as by-products of the manufacturing process or degradation of the buffer constituents may also be a problem. Therefore special procedures have to be adopted to remove them.

(iv) The buffer must not interfere with subsequent analytical measurements of either proteins or trace metals. In preliminary studies the interference effects of the buffer should be studied.

(v) If separation of the buffer from the fractions is required this should be accomplished in one step. For example, if the fractions are to be analysed by neutron activation then the choice of buffer is further complicated because it has to be removed before encapsulating the samples. For this purpose the acetate, carbonate or formate salts of ammonia could be used provided the trace metal speciation is no affected. Confirmation of this should be obtained by using a suitable biochemical buffer and another technique for the detection of the metal constituent.

The Effect of Ionic Strength

It is necessary to maintain the ionic strength of the separation medium close to the physiological value, especially when the metal-ligand interaction is predominantly electrostatic in nature. An increase in ionic strength decreases the strength of the metal-ligand interaction and the metal or ligand may be more vulnerable to substitution. Furthermore, increase in ionic strength may result in the precipitation of some species. In practice, an ionic strength of about 0.10 mol/L has been found to be adequate for most biological applications. The ionic strength of the buffer may be controlled by the addition of an adequate amount of chloride salt of sodium or potassium.

Temperature

Small increases in temperature may result in an increase in the rate of substitution and a shift in the equilibria of some reactions. Higher temperatures may lead to denaturation of large organic ligands, for example, proteins. As a consequence the solubility, immunological behaviour, and electrophoretic mobility of these ligands could be altered. On the other hand, low temperatures may slow down the enzyme activity and some labile complexes could remain stable. It is therefore worthwhile studying the effect of temperature especially when dealing with labile species.

The Effect of the Presence of Oxidising Agents

Most trace metals can exist in more than one oxidation state and complexes of the same metal in different oxidation states differ in their physico-chemical properties. Care

must therefore be taken to ensure that these oxidation states are not changed during the experiments. Consequently, the presence of oxidising agents must be carefully controlled. Not only is this necessary in order to maintain the oxidation state of the element but the presence of these agents enhance deteriorative reactions in biological systems which may also cause protein inactivation.

PROCEDURES FOR THE STUDY OF CHEMICAL SPECIES

Five distinct steps can be recognised in the procedures used for the study of the chemical forms of a given element: sampling, sample preparation, fractionation, detection and identification. From these arise eight points which should be considered when developing a scheme.

(i)   The effect of pre-sampling conditions and sampling techniques on the distribution and the nature of the chemical species of the element under study.

(ii) Sample pre-treatment before the separation step and how this may affect the speciation profile.

(iii) The amount of sample that can be fractionated without impairing the resolution of the separation technique.

(iv) The possible sources of trace metal contamination.

(v)   The form in which the fractions are obtained for example, either as liquid or solid.

(vi) The time required for a fractionation.

(vii) The possibility of protein denaturation and the possible loss of the trace metal before and during fractionation.

(viii) The sensitivity and selectivity of the detection techniques.

In the following sections the methods and techniques used for sample collection and pretreatment, fractionation, and detection of metalloid- and metal-containing species will be considered.

Sample Collection, Pre-treatment and Storage

Aitio et al., in this book, have discussed the various aspects of sample collection, pre-treatment and storage of biological samples. Most of the comments made regarding the precautions that must be taken in order to avoid extragenous and endogenous contamination are relevant to speciation studies. However, other additional precautions are necessary. These arise from the need to maintain the integrity of the metal-ligand interactions and to ensure that the complex three-dimensional configuration is not destroyed during any of the above steps.

Some of the changes in conditions that could have an adverse effect on the metal-ligand interactions and also lead to denaturation of proteins have already been discussed above. It is therefore essential that especially during the pre-treatment and storage steps changes in these parameters are kept to a minimum. Other factors that may affect the molecular configuration and hence cause the denaturation of biological molecules, have been reviewed by Kauzmann (1959), and Volkin & Klibanov (1989).

Fractionation Techniques

Introduction

Biological systems are a complex mixture of proteins, peptides, amino acids, inorganic complexes and ions. Because the systems are so complex it is virtually impossible to use a single fractionation technique to resolve the various constituents. However, complete resolution of the constituents is not a prerequisite in the study of trace metal bound fractions. Indeed, the extent to which the constituents are to be resolved by the separation technique is determined primarily by the selectivity and sensitivity of the available detection techniques.

A fractionation technique with low resolution coupled with a highly selective detection may be more powerful than a high resolution separation coupled with a relatively non-selective detection. In this regard, techniques that can be used specifically to determine the metal or a class of ligands are preferred to those that measure properties like the UV spectra or refractive index. Only in cases where the metal is associated with two partly unresolved species is the use of two or more fractionation techniques justified. It must be emphasised at this point that the chances of disrupting the metal-ligand association increase as the number of fractionation steps increase.

The constituents of a biological sample could be separated on the basis of differences in their molecular size, hydrophobic properties, charge, electromigration, solubility, partition coefficient, isoelectric point and immunological activity. Occasionally, separation by exploiting some of the above properties requires the use of experimental conditions which are not comparable to that found in the biological media and these changes may lead to the destruction of the metal-ligand interaction.

Another factor of importance in the choice of a fractionation technique is the sensitivity of the detection technique because the total concentration of some trace elements in biological samples lie at the limit of detection of most conventional techniques. Therefore fractionation methods in which the species are extensively diluted can only be used for these elements when it is possible to concentrate the fractions afterwards. Current technology enables analyte concentrations of about 1 μg/L to be determined in biological matrices. However, at these levels special precautions have to be taken to avoid contamination from external sources, and it is difficult to establish the accuracy of the determination. At present the speciation of metals or metalloids present at total levels less than 100 μg/L can only be undertaken with time-consuming and tedious methods which increase the probability of altering the metal-ligand interaction.

In the following sections some of the numerous fraction techniques that have been used for biochemical separation will be examined with particular reference to modification that could occur to the distribution of trace element-containing species.

Liquid Chromatography

The theory and practical applications of liquid chromatography have been the subject of numerous review articles (Barth, 1980; Regnier, 1983; Small, 1983) and books (Hamilton and Sewell, 1982; Krstulovic and Brown, 1982; Poole and Schuette, 1984) and therefore will not be discussed here. Instead, we will concentrate on the possible sources of contamination and the changes to the type and nature of the chemical species that may occur on the columns.

Sources of Metal Contamination

Conventional equipment used in column chromatography is generally not suitable for the fractionation of metal-containing species (Shih and Carr, 1981). Furthermore a number of studies (Trumbore et al., 1983, Sadek et al., 1985 and Sadek et al., 1987) have shown that the use of steel tubing, frits, and the presence of trace metals on the column packing material can lead to irreversible adsorption of proteins, and also affect the chromatographic retention processes. However, some manufacturers now offer alternative equipment with no metal components. Nonetheless, special precautions have to be taken to ensure that samples are not contaminated with the metals under study and it is helpful to obtain information about the materials from which the various components are manufactured and their stability in acid. The following precautions can help minimise contamination. (i) direct contact between the samples and metal components should be avoided; (ii) the tubings used should be made from polyethene, glass-lined steel, or an appropriate inert material; coloured rubber seals should be avoided; (iii) collect fractions in disposable polystyrene or polycarbonate vials with tight fitting polyethene caps. Tubes made from quartz glass or teflon may also be used; cleaning the tubes after every run may be tedious and time-consuming but this avoids the possibility of contaminating the subsequent fractions; (iv) components that are not vulnerable to acid attack should be decontaminated with a non-oxidising acid for at least 24 h and washed thoroughly with deionised water before use. Other components should be washed with small amounts of $(NH_4)_2H_2EDTA$ and then thoroughly rinsed with deionised water to remove any traces of the complexing agent.

Trace metal levels in the buffer and solutions used as eluent can be reduced to negligible levels by using the resin Chelex-100. Detailed procedures can be found in the literature (Rao et al., 1982). Furthermore, chemicals and reagents must be of the highest analytical purity. For some applications, AnalaR grade chemicals may not be suitable.

Other procedures for the removal of trace metal contamination from the separation matrix will be discussed under the individual separation technique.

Size Exclusion Chromatography

Separation of molecules by size exclusion chromatography (SEC) also known as either gel filtration or gel permeation chromatography, is accomplished by exploiting the differences in molecular dimensions. Soft gels that are hydrophilic in nature, and made

principally from cross-linking polysaccharide, dextran, agarose or polyacrylamide have been used for the separation of molecules of biological origin at low mobile phase velocities. Barth (1980) has summarised the properties of various commercially available packing materials used for SEC. For more recent developments in this field, up-to-date leaflets from the manufacturers should be consulted.

Although separation occurs by sieving or exclusion of the constituents by the column packing material, other so-called non-size exclusion effects are also known to occur. These effects, due mainly to the presence of charged residues on the column material, complicate the interpretation of a separation, and in the case of metal-containing molecules may lead to the alteration of the species. The non-size exclusion effects can be divided into two major groups: ionic interactions and adsorption. The extent of these interferences is determined in part by the type and process used in the manufacture of the column packing material, and in part by the experimental conditions.

The presence of ionised groups on the packing materials causes four types of problems:

First, any residual metal contamination from the buffer and from other sources is concentrated on the column. As a result ligands in the sample that form thermodynamically stable complexes with the metals will bind to them. It is also possible that non-specific effects such as local changes in ionic strength may lead to the elution of some of the metals with the sample. In either case new species are introduced into the fractions. Furthermore, the amount of metal associated with specific ligands may be over-estimated. Experiments by Woittiez (1984) have shown that zinc, copper, manganese and vanadium contamination on a column eluate with the protein fractions. This will doubtless lead to the over-estimation of the protein-bound fraction.

Second, the recovery of some metals will be severely affected, especially those that occur mainly in ionic forms. Johnson and Evans (1980) in a study of the binding capacity of soft gels from various manufacturers, found that between 30-570 μg of zinc and copper could be bound per gram of gel when water is used as eluent. Increasing buffer strength reduced, but did not eliminate the amount of analyte lost on the column. The data presented also indicated that the more highly cross-linked gels i.e. those used for the fractionation of low molecular mass fractions, have a greater tendency to concentrate these two metals. The extent of the interaction between metals and column materials is determined in part by the binding characteristics of the metals. For example, the ions of sodium iodine, rubidium, manganese and magnesium do not interact with the gel type Ultrogel AcA-34 whereas these of antimony, selenium, cobalt, copper, iron and zinc show varying degrees of affinity.

Third, if the conditions are favourable anionic groups can compete successfully with labile complexes for the metal so that the amount of metal associated with a particular ligand may be underestimated. If the exchange is total and irreversible the presence of a particular species in the original sample will be completely missed.

Fourth, the presence of heavy metals cations, for example Cd(II), Pb(II) and Hg(II) may lead to the inactivation of some enzymes (Vallee and Ulmer, 1972). The likelihood that an exchange of the bound metal between the labile complexes and the column increases

with the increase in separation times. Since more than 5 h is usually required to achieve reasonable resolution of the constituents of a biological sample when conventional soft gels are used, two reactions may occur:

$$ML_1 + L_2 \rightleftharpoons ML_2 + L_1 \tag{1}$$

$$ML_1 + L_2 \rightleftharpoons ML_1L_2 \tag{2}$$

where ML_1 is the complex in the sample and L_2 is an appropriately charged residue fixed to the column. Both ML_2 and ML_1L_2 therefore remain bound to the column. Note that the charges on the species have been omitted.

Another problem that may complicate the interpretation of the results is that some biological molecules have a tendency to dimerise in solution. Consequently, a new species may appear at high molecular mass region.

Ionic interactions can be reduced to negligible levels by increasing the ionic strength of the buffer or eluent solution. In any case, the ionic strengths of solutions used for speciation experiments should not be less than 0.30 (Herold, 1993). In cases where low recovery of the analyte is obtained in spite of an increase in ionic strength, the use of a different packing material should be considered or the ionised groups should be chemically modified. One chemical procedure developed by Bo Lönnerdal and Laas (1976) for agarose but also found useful for Sephadex involves the chemical reduction of the charged groups with sodium borohydride. Eaker and Porath (1967) have shown that a similar effect can be reduced by washing the gel with 1 mol/L aqueous pyridine. Additives used in chromatography to reduce these non-size exclusion effects should be used with care and experiments should be performed to show that an additive does not bind the metal under study.

Extensive discussions on non-size exclusion effects can be found in articles and reviews by various authors (Stenlund, 1976; Gelotte, 1960; Pfannkoch et al., 1980; Barth, 1980; Engelhardt, 1981; Belew, 1978 and Dubin, 1992).

Besides non-size exclusion effects, dilutions of the sample constituents during fractionation may lead to the dissociation of the labile complexes and thus affect the distribution of the metal. In order to reduce this problem some authors (Evans et al., 1978 and Yoza, 1977) have advocated the addition of a constant level of the metal or ligand in the buffer used to elute the sample from the column. This is not a very useful approach since even if the buffer does not form strong complexes with the free ion added new species may be introduced. The only sensible approach is to minimise dilution.

Both the time needed for a separation and the amount of dilution of the sample constituents have been drastically reduced with the introduction of high performance SEC (HPSEC). However, non-size exclusion effects tend to be more severe when chemically bonded phases are used. Fortunately, as the problems are identified and understood new column materials have been developed to replace the older ones. In a few articles (Cassidy, 1981; Drull, 1984) the usefulness of HPSEC for the fractionation of metal che-

lates have been demonstrated. Although this technique has been used for the fractionation of proteins and other biological molecules, few studies have been carried out with the sole intent of analysing the fractions for metal-containing constituents.

It is essential before the start of a separation that the metal contamination on the column packing material is removed. Three approaches can be applied: i) the use of complexing agents; ii) the use of dilute acid; iii) the use of purified buffer with ionic strength of about 0.1 mol/L. The first approach is the most effective. Complexing agents like EDTA with strong affinity for metals can be added in small amounts (0.001 mol/L) to the eluent solution. This should be followed by at least two bed volumes of eluent solution, without EDTA added, before the sample is applied to the column. However, a disadvantage of using this approach is that the presence of traces of complexing agent can be left on the column, and this can adversely affect the metal distribution.

In order to apply the second approach information on the stability of the gels at low pH values must be available. Most gels are stable at a pH of about 3, therefore the possibility of cleaning the gels at this pH value could be exploited. The conventional gels supplied already swollen should be deaerated and packed as recommended by the manufacturer. Four bed volumes of deionised water should be passed through the column followed by an equal amount of dilute hydrochloric acid at a pH one unit above the stability range set by the manufacturer. Finally the gel should be equilibrated with the buffer to be used. The pH of the effluent should be monitored until it is equal to the starting pH value.

Gels supplied in the dry form should be treated as follows: Clean a 1 L vacuum flask to be used for this procedure with (1 + 1) HNO_3 for 24 h and thoroughly rinse with deionised water before use. Add the required amount of dry gel to the flask and add 500 ml of an HCl solution at the required pH. Allow the beads to swell for 24 h at room temperature. Decant the supernatant and add a further 500 mL of acid solution. Repeat the above procedure twice. After decanting the supernatant for a third time add 500 mL of deionised water and allow the gel to settle. Decant the supernatant and repeat. Leave the gel overnight in deionised water and deaerate before packing the column. Equilibrate the column with the buffer to be used.

The above procedure can be applied mainly to conventional soft gels. With chemically bonded phases supplied already packed, it is preferred to run the buffer through the column until acceptable blank levels are obtained. Most pre-packed columns are made with components that are vulnerable to acid attack. Therefore changes in pH may result in their dissolution, and thus contamination of the effluent may result.

Application of Size Exclusion Chromatography

Size exclusion chromatography has been extensively used for the study of metal-containing species in various body fluids and tissues. A summary of some applications is given in Table 1. No attempt has been made to produce an exhaustive list, rather the examples have been chosen to illustrate the various procedures and precautions required when studying the distribution of the various chemical species of an element. More examples can be found in the chapters on the individual elements. Most of the applications reported provide only partial information on the species. For complete characterisation of a given chemical species a wide range of techniques are required, and these may not be available in a single laboratory. Besides the determination of the metal/metalloid con-

TABLE 1

APPLICATION OF SIZE-EXCLUSION CHROMATOGRAPHY TO THE STUDY OF THE
SPECIATION OF VARIOUS METALS

Element	Additional Techniques Used	Sample	Reference
Al	Graphite furnace atomic absorption spectrometry (GFAAS)	Human serum from patients on dialysis	King et al. (1982)
	GFAAS, Ultrafiltration	Human serum from patients on dialysis	Gardiner et al. (1984)
	GFAAS	Human serum	Leung et al. (1988)
As	GFAAS	Human urine	Chana and Smith (1987)
Au	GFAAS, Electrophoresis, Polyethene glycol precipitation	Sera from patients undergoing chrysotherapy	Kamel et al. (1977)
Cr	GFAAS	Pooled lyophilized animal serum	Graf-Harsanyi and Langmyhr (1980)
Cu, Fe, Zn	GFAAS, Immunonephelometry	Amniotic fluid	Gardiner et al. (1982)
Cu, Cd, Zn, Se	Neutron activation analysis (NAA)	Human liver	Nordheim and Steinnes (1975)
Cu, Fe, Zn, Al, Mn	NAA, UV detection	Human serum	Fritze and Robertson (1968)
Cu, Br, Mn, V, Se, Rb, Fe, I, Zn	NAA	Human serum	Woittiez (1984)
Mn	Tracer technique, equilibrium dialysis	Human or rabbit plasma	Nandedkar et al. (1973)
Ni	Tracer studies, polyacrylamide gel electrophoresis, ultrafiltration	Human serum	Sunderman et al. (1983)

TABLE 1 (continued)

Element	Additional Techniques Used	Sample	Reference
Ni	GFAAS	Helicobacter pylori	Hawtin et al. (1991)
Se		Human serum	Borglund et al. (1989)
		Human serum	Butler et al. (1991)
Zn	Ultrafiltration, ion-exchange chromatography NMR, IR	Human milk	Bo Lönnerdal et al. (1980)
	Affinity chromatography, GFAAS, Kinetic immunoturbidimetry, Radial immuno diffusion.	Human serum	Foote and Delves (1984)
	GFAAS, isoelectric focusing	Human erythrocytes	Gardiner et al. (1984)

tent and approximate molecular mass of the complexes, the identification of the ligands, the oxidation state(s) of the metal, and possible binding sites of the metal are all necessary information. In addition, investigations carried out under physiological conditions to determine the reactivity of the species may also be valuable.

The molecular mass of ligands associated with metals in biological samples can range from below one hundred to over a million. Consequently, it is not possible to use one gel type to fractionate the various constituents. Hence, before any separation is undertaken the molecular mass range to be investigated should be defined. This would help not only in the choice of the gel type but also in the selection of the method for ligand detection.

An advantage of using liquid chromatography for fractionation is that the column can be directly coupled with some analytical instruments, and/or the fractions can be collected and analysed by techniques which are not amenable to this type of marriage. Direct coupling results in time-saving and frees the investigator for other tasks. Most SEC columns are usually coupled with a UV detector set at a wavelength of 206, 254 or 280 nm in order to monitor the elution profile of proteins, polypeptides, nucleotides etc. Atomic absorption and plasma emission instruments have been directly coupled with the columns in order to provide simultaneous information on the metal distribution profile. These techniques and their use in this mode will be discussed later.

Although the determination of the metal or metalloid is relatively straightforward, the complete identification of the ligand(s) presents some challenge. A systematic approach is required if the ligands are to be completely characterised. As a first step after the separation of the constituents, the column should be calibrated with compounds of known molecular mass. Calibration kits are obtainable from most manufacturers, however, if

compounds are not available in the molecular mass range of interest well-characterised compounds could be used. Secondly, from the information gained from the calibration and other experiments, for example, those to determine the isoelectric points of the molecules, biochemical reference books could be consulted and a list of compounds that fit the available data should be made. At this stage specific analytical techniques can be applied in order to identify the probably compound.

Ion Exchange Chromatography

Differences in the net charges of the species are exploited to achieve separation by ion exchange chromatography (IEC). The packing material used for the column is composed of ionisable functional groups attached through covalent bonds to an insoluble matrix. The functional groups are in equilibrium with mobile counterions (cations or anions depending on the character of the functional group). On applying a sample to the column, the constituents compete with the counterions for the available groups, and if the experimental conditions are favourable the charged species are preferentially bound. Selective elution of the bound species is achieved by altering either the pH and/or ionic strength of the eluent in a predetermined manner. In an excellent article, Rabel (1979) has discussed the various considerations in the choice of equipment and experimental conditions.

One of the factors that has limited the use of IEC for the fractionation of crude biological samples has been the risk of irreversible interaction between the constituents and the packing material. This type of interference has been partly solved by introducing precolumns and the design of new packing materials. In clinical chemistry, IEC has found extensive use for the separation of amino acids, and the isolation of proteins in relatively pure samples.

In using IEC for the study of the distribution of the various chemical species of a given element, two problems have to be considered: (i) the sources of contamination; (ii) the stability of the metal-ligand association. The sources of contamination in IEC will generally be similar to that in SEC. However, with this technique the possibility of contamination is further increased because of the increased number of binding sites. The column can be decontaminated by the procedures outlined for SEC. In addition, the reagents used must be very pure and free of the metals under study. At the moment very little is known about the fate of metals bound to ligands that are subjected to IEC. The interaction in metal-proteins as against metalloproteins involve principally electrostatic interaction between the metal and protein. Consequently, small changes in ionic strength or pH would have a drastic effect on the association. An increase in ionic strength may result in greater competition between the ions and metal for the binding sites on the ligand. Changes in pH can alter the acid-base equilibria. All these factors can contribute to the dissociation of the metal from the ligand and metals associated with metalloproteins could be rendered vulnerable to substitution if the changes in ionic strength and pH are large.

It is therefore to be concluded that the IEC displacement of the metal from the protein could occur, and this should be borne in mind when applying this technique to trace metal studies. However, in examples of applications (Veening and Willeford, 1983; and Nickless,

1985) of IEC is has been shown that this technique could be successfully applied to the separation of metal chelates. Anion-exchange chromatography is now widely used for the study of the distribution of arsenic species in a number of biological samples (Morita et al., 1981; Spall et al., 1986; Heitkemper et al., 1989).

A variant of IEC is ion-chromatography. In some applications it has been used for the determination of inorganic ligands. Examples of the application of this method to biological samples can be found in works of Smith and Chang (1983) and Small (1981).

Affinity Chromatography

Separation by affinity chromatography utilises immobilised adsorbents which can bind specifically and reversibly to complementary molecules. A suitable substance for the solid support material constituting the stationary phase is agarose, which exhibits minimal adsorption, maintains good flow rates after coupling and tolerates extremes of pH, ionic strength as well as high concentrations of chaotropes such as 7 mol/L urea or 7 mol/L guanidine HCl (Freifelder, 1976). The ligands (adsorbents) must possess a functional group that can be modified for attachment to the solid support and another binding site with adequate affinity for the molecule to be purified: equilibrium constants K_L or the order 10^{-4} to 10^{-8} mol/L are satisfactory for most separations. The immobilised ligands may be either specific for individual substance or group specific for a number of chemically similar molecules. The application of affinity chromatography to the separation of proteins has been discussed by Strosberg (1986). One group specific ligand which has been used for the successful separation of a range of enzymes and for the separation of albumin from other zinc binding ligands is Immobilised Cibacron Blue F3G-A (Turkova 1984) for which the formula is:
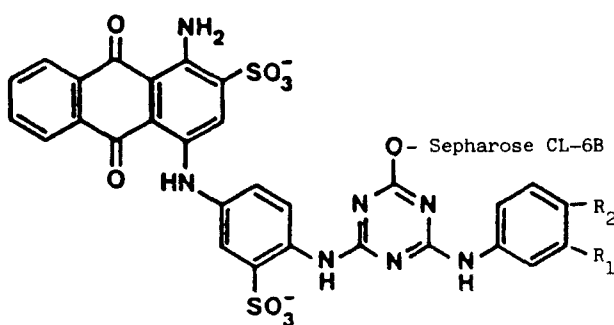

Fig. 1. The Structural Formula of immobilised Cibacron Blue F3G-A.

Binding of the immobilised dye to the separated molecules is thought to occur via the free amino group which binds to NAD(H) and NADP(H) binding sites in the proteins — so-called dinucleotide fold — since the ligand has poor affinity for albumin following CNBr coupling which makes this amino group unavailable (Travis et al., 1976). The sulphate

groups of the dye allow some cationic interaction with proteins and at acidic pH (5.0) unbound proteins elute from the resin in order of increasing pI (Gianazza and Arnaud 1982 a). In applications of affinity chromatography to separation of metal-binding species it is important to consider whether the interactions of the species with the immobilised dye, either by binding via the amino group or by ion exchange via the sulphate groups, produce any disturbances of metal-protein interactions.

Hughes et al. (1982) showed that 200-2000 μmol/L concentrations of first row transition ions promote the binding of proteins to immobilised triazine dye affinity adsorbents, including Cibacron F3G-A. With some molecules the binding effect was almost totally specific for both the metal and the dye e.g. avolbumin-Al(III)-Cibacron Blue F3G-A (or Procian Orange MX-G) whereas with others such as carboxypeptidase, although Zn(II) was the most efficient at promoting dye binding other ions were also effective in the order Zn(II) > Co(II) > Mn(II) ~ Ni(II) > Cu(II). The ionic concentrations associated with efficient protein binding were, however much higher than would be encountered in body fluids. At low ionic strength and at lower concentrations of zinc (5 μmol/L) Cibacron Blue F3G-A has been shown to bind more than 60% of added Zn(II) but there was no detectable zinc binding (10±2.6% recovery of added Zn(II)) from a pH 7.4 buffer containing 0.05 mol/L NaCl in 0.05 mol/L Tris-HCl, Foote and Delves (1983). These workers showed a lack of affinity of the resin for Zn(II) ions under conditions used for separating albumin from other zinc binding ligands.

The possible dissociation of metal-protein complexes during separation is another potential source of error in speciation studies. Smith et al. (1979) applied [65]Zn-rat albumin complex to a Blue Sepharose column and recovered 94% of the [65]Zn still in association with albumin. Foote and Delves (1983) carried out a detailed study of the dissociation of Zn-albumin separated from human serum on Sepharose Blue Cl-6B which contained 2.5 mmol/L dye settled gel and showed that the nature of the buffer solution determined the degree of dissociation. Adsorption of albumin from a buffer solution containing 0.05 mol/L NaCl plus 0.05 mol/L phosphate at pH 7.4 was accompanied by significant dissociation of the Zn-albumin complex. Of the zinc initially bound to albumin, only 68% was retained by the protein and 28% eluted directly with the buffer solution. The overall recovery of zinc of 96±2% excluded exogenous contamination or loss of the metal. However, using a buffer solution containing 0.05 mol/L NaCl plus 0.05 mol/L Tris-HCl at pH 7.4, less than 2% of the zinc eluted with the buffer and 99±2% of zinc originally bound to albumin was retained by that protein. Clearly, the relatively labile zinc-albumin binding was not affected by the dye-protein interaction in the NaCl/Tris-HCl buffer. The degree of dissociation of Zn-albumin binding during elution of the adsorbed protein was also affected by the buffer solution. Elution with 0.05 mol/L sodium octanoate gave 97±2% recovery of albumin but with complete dissociation of all bound zinc which was retained by the column and which required EDTA for its removal from the denatured column. Elution with 0.2 mol/L NaSCN at pH 7.4 gave 84±1% recovery of albumin without any dissociation of the zinc-albumin complex and without denaturing the column.

The negligible affinity of Blue Sepharose for Zn(II) ions and the lack of any significant dissociation of Zn-albumin complexes during adsorption and desorption make this resin

attractive for studies of zinc species in biological fluids. The distribution of zinc among human serum proteins using this technique was found to be 81±3% bound to albumin and 19±3% bound to α_2-macroglobulin (Foote and Delves, 1983). These data are in agreement with distribution data reported by others using relatively mild procedures unlikely to produce disturbances of the zinc binding equilibria e.g. polyethene glycol precipitation (Giroux et al. 1976), constant ionic strength gel filtration (Chilvers et al., 1984). The range of proteins separated by Sepharose gel affinity resin chromatography may be extended by increasing the amount of dye incorporated into the agarose gel. For example, Gianazza and Arnaud (1982b) have used gels incorporating 4.5 mmol/l dye of settled gel to retain haptoglobin, α_2-macroglobulin etc., and have identified 27 different plasma proteins after fractionation on Cibacron Blue F3G-A.

Reversed-Phase Chromatography (RPC)

Reversed-phase chromatography is now widely used for the fractionation of biological molecules. The technique is based on the use of a non-polar stationary phase and a polar mobile phase. The stationary phase is usually made of a hydrocarbonaceous layer, either *n*-octyl, C_8, or *n*-octadecyl, C_{18}, ligands, chemically bonded to the surface of a silica matrix via siloxane bonds. Separation is achieved by exploiting the difference in the hydrophobic properties of the molecules.

A number of articles (Karger et al., 1976; Colin et al., 1983; Verzele and Dewaele, 1984) have examined the mechanisms of retention on the column. Hydrophobic, silanophilic and solvophobic interactions have been identified as important factors in the retention processes. It is likely that some or all of these interactions exist to varying degrees during a separation and the dominance of any one will depend on the experimental conditions.

Reversed-phase chromatography offers excellent resolution for biological molecules, and it is particularly useful for the separation of species of a homologous series. However, a drawback of the technique is that the strong interactions between the solutes and the stationery phase could lead to denaturation of large biomolecules with the consequent loss of biological activity. It is therefore essential that the separation conditions are chosen such that the biological activity of any species under study is preserved. In most applications, it is advisable to perform a preliminary pre-treatment in order to remove any likely interferents before applying the sample on to the column. This is necessary so that the column is not fouled with constituents that are either irreversibly bound or too large to go through the column.

Presently, RPC is used as part of the fractionation protocol for the study of organometallic species. In a number of applications, the species are fractionated after an appropriate derivatization step. Some examples of the application of RPC for the study of the speciation of various elements are: As (Francesconi et al., 1985), Cr (Krull et al., 1983), Pb (Robinson and Boothe, 1984), Sn (Krull and Panaro, 1985).

Gas Chromatography

In contrast to liquid chromatography which is the technique of choice when conside-
ring high molecular mass constituents, gas chromatography (GC) is well-suited for the
fractionation of low molecular mass species which are volatile, thermally stable and prefer-
ably neutral. Some species that do not already possess the above properties can be
converted into forms that are amenable to GC. Various derivatization procedures that can
be used to perform such conversions have been described by Poole and Schuette (1984).
However, it is worth mentioning that hydride formation and alkylation are the two most
commonly used derivatization methods that have found application in the study of the
speciation of various elements like As, Bi, Ge, Hg, Pb, Sb, Se, Sn, Te and Tl. Two points
have to be considered when derivatization is performed: first, the specificity of the chemi-
cal conversion; second, the percentage yield of the reaction. A specific reaction is
desirable in order to avoid the introduction of artefacts. Moreover, if a quantitative esti-
mate of the amount of the original species is required, then it is essential that the extent of
the conversion is known.

Because of the detrimental effect of the presence of water on the column substrate,
and the risk of irreversible interactions of some matrix constituents with the column
material, direct application of an untreated biological sample is not advisable. Conse-
quently, a sample pretreatment step is always required. This step may involve the removal
of most of the matrix constituents by ion-exchange chromatography, ultrafiltration and/or
dialysis followed by solvent extraction and derivatization of the species under study. The
complexity of the method chosen would depend on the stability of the species and the
extent to which other matrix constituents interfere with its isolation.

Various species of the elements lead, mercury, arsenic, selenium, thallium and tin that
are of environmental and clinical significance have been determined in biological materials
(see the individual chapters in this book). The application of gas chromatography to the
determination and identification of inorganic compounds, metal complexes and organo-
metallics has been reviewed by Uden (1985). Some of the methods from the literature
reviewed by the author could be applied to the distribution of chemical species in biologi-
cal materials after appropriate sample preparation. Recently, Chau and Wong (1989) have
reviewed the application of gas chromatography to the study of the chemical species of
various elements in clinical and environmental samples.

Besides the universal detector systems, for example electron capture, flame ionisation
and thermal conductivity usually coupled with gas chromatographic columns, various
other detectors are now being used to provide specific information. For example, the gas
chromatograph/mass spectrometer couple has been used for structure elucidation of the
separated fractions. The mechanics of this hybrid technique have been described by
Message (1984). Other techniques used to detect the metal and/or metalloid constituents
include inductively coupled plasma spectrometry and atomic absorption spectrometry.
Ebdon et al. (1986) have reviewed this mode of application. The type and mode of
combination of the detectors depend on the ingenuity of the investigator. Krull and Driscoll
(1984) have reviewed the use of multiple detectors in gas chromatography.

Selective Protein Precipitation

Among the earliest methods of protein fractionation is the selective precipitation of fractions by the addition of neutral salts or organic solvents. Compounds used to effect such precipitation include chloroform, ammonium sulphate, sodium sulphate, ethanol, and more recently ethodin (Rivanol) and polyethene glycol. A discussion of the application of these compounds can be found in a book by Curling (1980). In order to precipitate a given fraction, the concentration of the salt or solvent, the pH, ionic strength and temperature of the sample solution must be carefully controlled. Even with this control, the method can yield only limited resolution between the fractions. This method does however provide a rapid and useful separation when it is known that a trace metal is associated with two major fractions one of which could be precipitated. An example of this is the study of zinc distribution in serum using polyethene glycol (PEG). In human serum zinc is known to be associated principally with two major fractions, α_2-macroglobulin and albumin. The protein α_2-macroglobulin is precipitated in the presence of PEG and therefore the amount of zinc bound to this protein can be estimated by difference from total zinc present (Giroux et al., 1976). Undoubtedly, the distribution of other trace metals could be similarly studied.

Although proteins are precipitated at a critical PEG concentration the mechanism by which this occurs is not well understood. Laurent (1963a,b) has proposed that for precipitation to occur the polymer must sterically exclude the protein from part of the solvent and in so doing it brings the protein to its solubility limit. According to this hypothesis large proteins will be readily excluded and therefore easier to precipitate. Although Chesebro and Svehag (1968) agree that in general the heavier proteins are precipitated first they contend that this process does not proceed strictly in the order of molecular mass. This suggests that other unknown factors are also involved. Polson et al. (1964) have suggested that the amount of PEG required to precipitate a protein depends on the charge on the protein at the pH of the medium.

Precipitation of α_2-macroglobulin with PEG does not appear to denature the protein to an extent that zinc is lost. An indication that PEG could be used to study some metalloproteins. If the removal of PEG from the precipitate is required this can be easily accomplished by redissolving the precipitate in buffer and subjecting the solution to SEC.

Selective precipitation of protein provides a simple, fast and cheap method that could be used to fractionate proteins. However, this can be applied only when there is evidence that the distribution of the chemical species of the element is not affected during the process.

Ultrafiltration and Dialysis

Ultrafiltration and dialysis are two techniques that have been used extensively for the separation of small molecules from biological samples. Although both are based on the permeation of the molecules through a semi-permeable membrane, the condition under which separation is achieved are essentially different. With ultrafiltration the solution containing the constituents to be fractionated is passed under hydraulic pressure through a

membrane filter which retains molecules with dimensions greater than the pore size. On the other hand, separation by dialysis is achieved by allowing the molecules with dimensions smaller than the pores of the membrane to diffuse through under the influence of a concentration gradient into another solution, that does not contain the species to be separated.

Each filter is characterised by its retention cut-off value. This means that for a given filter constituents with molecular mass above this value will be retained. Although separation is achieved by exploiting the differences in molecular dimensions (size and shape) and cut-off quoted by most manufacturers is given in molecular mass. This is misleading because solutes with the same molecular mass but with different shapes may be retained to a different extent. Another fact usually ignored by most investigators is that there is a given tolerance in the cut-off value. This tolerance is determined by the properties of the membrane material, the nature of the solution to be separated, and the experimental conditions.

Several factors can affect the retention properties of the membrane and some of these will be discussed here. During ultrafiltration the transport of solute to the surface of the filter is faster than the rate at which permeation through the membrane occurs. This is further complicated, as ultrafiltration progresses, by an increase in the concentration of retained molecules at the membrane. Both events contribute to the phenomenon called concentration polarization. This effectively introduces a second layer of 'membrane', and as a consequence the retention characteristics of the system are altered. The build-up of solute can be reduced by introducing some form of agitation at the filter surface. However, this procedure does not seem to be effective against the gel-type layers formed by proteins. Various procedures have been suggested to slow down this build-up of solute: the solution can be diluted with an appropriate solvent; the ultrafiltration process can be interrupted and the flow reversed momentarily; a low operation pressure could be used.

Retention is also affected by the adsorption of constituents on the membrane. If this occurs the membrane should be exchanged for another manufactured from a different polymeric material.

The retention characteristics are also influenced by the net residual charge on the membrane. In the absence of other complicating factors, like those already referred to above, it would therefore be expected that molecules or ions with the same charge sign as that on membrane will be rejected. However, in complex solutions containing many charged species, the presence of charged constituents that do not permeate the membrane may enhance the permeability of other species with similar charge. An additional source of interference is the Donnan membrane effect.

The effect of the charged surface is closely related to the pH and ionic strength of the solution. For example, if the pH of the solution corresponds to the isoelectric point of a protein its net charge will be zero and as a result it will be transported unimpeded through a charged membrane. Decrease in ionic strength due to the loss of electrolytes from the solution during ultrafiltration results in an increase in interaction between the species themselves and with the membrane. Some of the above mentioned effects have been studied and discussed by Staub et al. (1984).

The effects described above also occur to varying degrees in dialysis although the experimental conditions are different. A detailed discussion on the use of dialysis to separate molecules, as well as determine the stability constant of complexes can be found in books by Berg (1963), and Hwang and Kammermeyer (1975).

Thus far no consideration has been given to the changes that could occur to the form and nature of the metal-containing constituents. Changes caused by the presence of a net charge on the membrane would be similar to those described for size exclusion chromatography. Parameters that are likely to change during ultrafiltration and dialysis and also influence the conditional stability constants of the equilibria involving metal-containing species include pH, ionic strength and volume.

In humans, the pH of extracellular and interstitial fluids is maintained by the dicarbonate buffer system (H_2CO_3/HCO_3^-). Under the conditions used for ultrafiltration some CO_2 is lost to the gas phase, and consequently the pH of the solution is altered. The changes that could occur to the distribution of the various metal-ligands due to pH changes have been already discussed. In order to keep the pH constant a mixture of 95% O_2 and 5% CO_2 could be used as the pressure gas.

For speciation studies it is important that the volume change is kept at a minimum, preferably less than 10% of the starting volume. Larger volume changes may result in a shift in the equilibria of labile complexes and as a consequence new species may be formed. A practical reason for keeping the volume change to a minimum is that the severity of some of the problems already outlined is reduced.

Separation of molecules with widely different molecular weights can be achieved by using the appropriate filters in parallel or in series. In the series mode or tandem arrangement, the problem caused by concentration polarization is at its most severe at the membrane in direct contact with the starting solution but decreases at subsequent filters.

Comments made under the section on liquid chromatography as regards the sources and prevention of trace metal contamination also apply to ultrafiltration and dialysis.

Although ultrafiltration is a cheap and fast method, the interpretation of the results is complicated by the effects described above.

Species Detection, Identification and Characterisation

The presence of a trace element-containing species is usually detected by applying analytical techniques that determine the metal or metalloid constituents. The methods and techniques for such determinations have been adequately dealt with in various chapters of this book. No additional comments are required other than to remark that in cases where the concentration of the analyte is at the limit of detection of the analytical technique a preconcentration step (Poole and Schuette 1984) should be considered. This step could involve sample evaporation, solvent extraction, dialysis, ion-exchange chromatography and/or electrolytic preconcentration. If further analytical procedures are contemplated, for example, characterisation of the species, then it is essential that the species is not destroyed during the preconcentration step.

The use of a fractionation technique coupled with the determination of the metal or non-metal content could provide some information about the species under study. This information may include the approximate molecular mass, isoelectric point and electrophoretic mobility. If well-characterised standards are available with similar properties as the species of interest, identification is possible. In practice, however, because of the very limited number of well-characterised standards complete characterisation and identification is only possible from first principles. Indeed, complete characterisation may not, in some cases, be necessary for the interpretation of the experimental results. Partial characterisation may be all that is needed. Some of the information that may be required and the techniques used are summarised in Table 2. A detailed account of some of the approaches used for structure elucidation and the study of the reactivity of various trace element containing biological molecules can be found in the book by Hughes (1981).

COMBINATION OF ANALYTICAL TECHNIQUES

In recent years, developments in analytical instrumentation have made it possible to couple two or more instruments in a bid to maximise the amount of information that could be acquired from a sample. Where these attempts have been successful these have resulted in a more efficient use of the sample material, drastic reduction in waiting time, minimum sample manipulation, and in relation to trace metals the sources of external contamination have been reduced.

However, in order to couple instruments successfully three requirements have to be fulfilled. First, the interface must be simple, inexpensive and must not degrade performance. Second, the time scale in which the instruments operate must be compatible. Third, the combination must offer some advantage over the use of the individual components.

Recently, the application of coupled instruments to the study of chemical speciation in biological samples has grown rapidly. It is noteworthy also that various combinations have been used in related fields. Wilkins (1983) has reviewed the developments in hybrid techniques used for the analysis of complex organic mixtures. The approaches described could be used to identify and characterise metal-containing volatile species. In a detailed article, Krull (1984) has discussed the various combinations suitable for the detection of constituents in the effluents from high performance liquid chromatography. Some of the procedures described can be used for the study of metal-containing species in biological samples after slight modification. Ebdon et al. (1987) have reviewed the application of directly coupled liquid chromatography with atomic spectrometry. A sign that this type of combination of techniques is now commonplace, is evident in the fact that a recent book edited by Harrison and Rapsomanikis (1989) is completely devoted to the topic of interfacing chromatography with atomic spectroscopy. The last five years have seen the emergence of inductively coupled plasma-mass spectrometry systems coupled to high-performance liquid chromatography as powerful tools for the separation and detection of various chemical species. A number of workers (Thompson and Houk, 1986; Dean et al.,

TABLE 2

THE INFORMATION AND TECHNIQUES REQUIRED FOR THE COMPLETE
CHARACTERISATION OF METAL-CONTAINING SPECIES

Information Required	Techniques and Methods	Further Reading
Trace metal content	Plasma emission spectrometry	Thompson and Walsh (1983)
	Atomic absorption spectrometry	Delves (1981, 1987)
	Neutron activation analysis	Heydorn (1984)
	Anodic stripping voltammetry	Wang (1985)
	ICP-MS	Vela et al. (1993)
Metal containing volatile species	Gas chromatography/mass spectrometry	Grevers (1985)
	Gas chromatography/atomic absorption spectrometry	Harrison (1985)
Oxidation state of the metal	Anodic stripping voltammetry	Vydra et al. (1976)
	Electron pair resonance (EPR)	Wertz and Bolton (1972)
	Mossbauer spectroscopy	Dickson (1984)
Environment of the metal	EPR	Leyden and Cox (1977)
	Nuclear magnetic resonance (NMR)	
	Electron energy loss spectroscopy	
	X-ray techniques	Matthews (1977)
Identification and characterisation of proteins and other large molecules (M.M. 10,000)	Ultraviolet-visible detection	
	Isoelectric focusing	
	Enzyme activity	
	Immunochemical methods	Clausen (1969)
	Sequence analysis,	
	Optical rotatory distortion	Jones (1976)
	NMR	Sadler (1986)
	X-ray techniques	
Identification and characterisation of amino acids and peptides	Amino acid analysis	
	Sequence analysis	Winter (1986)
	Laser Raman spectroscopy	
Identification of anions	Ion chromatography	Smith and Chang (1983)
	Atomic absorption spectrometry	Garcia-Vargas et al. (1983)

TABLE 2 (continued)

Information Required	Techniques and Methods	Further Reading
Stability constants of the complexes	Equilibrium dialysis Titration Ultrafiltration Size exclusion chromatography	Yoza (1977)

1987; Beauchemin et al., 1988; Heitkemper et al., 1989; Matz et al., 1990) have already demonstrated the usefulness and versatility of this combination.

CONCLUSION

In order to obtain results that accurately reflect the *in vivo* distribution of trace element containing species, it is necessary not only to appreciate the limitations of the techniques and methods used but also it is essential to have a thorough understanding of the chemistry of the element and the system under investigation. With the technology presently available, the distribution of various species of some trace elements in a wide variety of samples has been accomplished, however, for other elements this has been difficult if not impossible. Progress in the research of this latter group of elements must await further developments in ultra-trace analytical techniques.

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Chapter 10

Interlaboratory and intralaboratory surveys

Reference methods and reference materials

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INTRODUCTION

Interest in the roles of both essential and non-essential trace metals in human health and disease has undergone an enormous expansion in the last thirty years. This has come about partly due to major advances in our knowledge of inorganic biochemistry (Frausto da Silva and Williams, 1991), as well as the wider introduction into clinical laboratories of powerful analytical techniques such as graphite furnace atomic absorption spectrometry (Delves, 1987; Slavin, 1988). Developments in instrumentation and chemical matrix modification techniques have also brought about dramatic improvements in analytical performance (Delves, 1987; Baruthio et al., 1988; Slavin, 1988; Christensen et al., 1988; Savory and Wills, 1991). Other analytical techniques, such as inductively-coupled plasma emission spectrometry (ICP) and ICP-mass spectrometry are also finding wide application in the clinical analysis of trace elements (Kimberly and Paschal, 1985; Delves and Campbell, 1988; Melton et al., 1990). Although the cost of such instruments tends to restrict their use only to specialist centres, they have very important roles as reference techniques in the characterisation of reference materials (Delves and Campbell, 1988).

In the last decade, there has been a marked increase in the toxicological and clinical demand for trace element analysis which has been reviewed by a number of authors (Delves, 1987; Kruse-Jarres, 1987; Versieck and Cornelis, 1989). This places big demands on the reliability of such analyses and highlights the importance of quality control in the determination of trace elements (Boyd, 1983; Brown, 1982; Inhat et al., 1986a,b; Delves, 1987; Ihnat, 1988; McKenzie and Smythe, 1988; Versieck and Cornelis, 1989; Brown, 1991). Early interlaboratory comparison studies revealed that there were serious difficulties in achieving precise and unbiased quantitative measurements of trace metals in biological materials. Moreover, reported 'normal' concentrations of some trace metals varied by several orders of magnitude when results from so-called specialised laboratories were compared (Versieck, 1984; Ihnat, 1988; Versieck and Cornelis, 1989). In the last

decade however, strenuous efforts have been made towards the harmonisation of trace metal analyses (Brown, 1982; Ihnat, 1988; Versieck and Cornelis, 1989). As part of this process much attention has been paid to the dual problems of imprecision and of bias control (Aitio, 1981; Taylor, 1987). The development of definitive or reference methods and certified or "surrogate" reference materials has also been an important part of this harmonisation process. In many ways this is following on from the developments which took place in the field of clinical biochemistry more than a decade earlier (Rand et al., 1980).

POTENTIAL SOURCES OF ANALYTICAL ERROR

The realisation that every laboratory determination that is carried out is associated with both random and systematic errors has had a major impact on laboratory medicine in the last thirty years. It is also at the heart of quality control and quality assurance procedures which are primarily concerned with understanding the sources of such errors and their suppression or minimisation (Whitehead, 1977; Aitio, 1981; Taylor, 1987). However, it has been pointed out by Broughton (1983) that all laboratories may carry out some form of "quality control" but this is often designed to give retrospective reassurance rather than provide prospective action. The dual concepts of bias and precision in laboratory medicine are well known, but not always appreciated even by users of reference materials (Taylor, 1985; Taylor, 1987). By definition an unbiased result should be the "true" result, but in practice this is hardly ever achieved. The nearest approach to a true value is generally obtained by using a certified reference material and a definitive method, but these ideals are unobtainable in the case of most trace metal analyses.

The potential sources of error in carrying out the determination of trace metals in biological material are legion and should never be underestimated. Some of these sources of error have been discussed by previous authors and have also been the subject of extensive reviews (Nieboer and Jusys, 1983; Versieck et al., 1982; Versieck, 1984; Versieck and Cornelis, 1989). Some of the most important potential sources of error in trace metal determination are discussed in outline as follows:

Volumetric measurement of liquids

As pointed out by Yeoman (1983), the simplest yet one of the most important sources of random error is in the volumetric handling of liquid samples or standards. Differences in the surface tension and viscosity of specimens, calibration solutions or controls may cause major problems in analytical performance. The usefulness of lyophilised control material can often be abrogated by inaccurate reconstitution or unforeseen contamination. Any pipettes (manual or automatic devices) used in volumetric measurements should be of grade 'A' quality and regularly checked for bias; they must be carefully washed and kept clean, free of contamination.

Collection and sampling of tissues or solids

This is a difficult and complex source of error (Stoeppler, 1983; Versieck and Cornelis, 1989). The water content of tissues is quite variable and depends on the nature of the tissue sampled and the condition of sampling. Great care and experience is required in obtaining a properly representative sample of a tissue and consideration needs to be given to the anatomical site(s) of sampling. In some cases, there may be important regional differences in the distribution of elements in an organ (Raithel and Shaller, 1990). The 'dry' weight of tissues is often used in order to obtain a more accurate estimate of the tissue mass; however, additional errors may be introduced during the drying process. Some metals may be lost, or change oxidation state, other metals may be lost so as to corrupt the initial balance of metal speciation present before drying. The collection process itself e.g. the use of metallic instruments may also be a potent source of contamination for those metals present in ultra-trace quantities.

The expression of results for cellular components is particularly difficult to resolve. It is possible to express results in terms of cell number, dry weight, total protein or DNA content. Each method of expression may be prone to certain errors, particularly when assessing the influence of disease states or malnutrition (Patrick and Dervish, (1984).

Instrument characteristics and variation during analysis

There are several sources of random and systematic error that can be attributed to instrument characteristics and variation during an analytical run. Although graphite furnace atomic absorption spectrometry is the most widely used and respected technique in trace metal determination, the complex chemical and physical processes involved in the various stages of drying, ashing and atomisation are far from understood (Frech et al., 1982). Changes in furnace design or the method of background correction may cause important differences in analytical performance (Slavin, 1988). Careful attention should be paid to optimising instrument performance and to the use of frequent and appropriate "control procedures" to ensure acceptable bias and precision (Delves, 1987). Methods developed on one manufacturer's instrument may not always be directly transferable to another manufacturer's instrument. Subtle differences in furnace performance may cause dramatic differences in recovery and precision. Atomisation from the wall of a graphite tube may be considerably different from that obtained using a platform technique (Delves, 1987; Slavin, 1988). The use of oxygen ashing or other matrix modification techniques may cause important differences in the amount of interfering background present. The analysis of a wide range in biological materials for the same element can present a difficult and demanding task, as in the case of aluminium (Delves et al., 1989).

Method of calibration and bias control

Although a routine aspect of any analytical procedure, the method of calibration is a major potential source of systematic error. Error in the preparation of calibration standards or their working dilutions is one obvious source of discrepancy. The recovery of an

element obtained from an aqueous standard may be different to that obtained from biological specimens. Great care is required in the method of calibration procedure so as to minimise any 'matrix' effect; there can be important limitations in the casual use of "standard additions" techniques (Slavin, 1988). An important check on bias is a good agreement between different calibration procedures, using both standard additions and direct determination with matrix matched standards. Simple differences in the viscosity of liquids may also cause large analytical errors. The use of surrogate or certified "reference materials" and common internal control materials can be a simple yet effective means of minimising interlaboratory errors. This technique has been successfully applied to the improvement in laboratory performance of trace metal assays in national and international screening programs (Berlin et al., 1983; Department of Environment, 1983; Taylor, 1988a; Vahter and Friberg, 1988).

Contamination

Contamination is one of the most important and persistent sources of both random and systematic error in trace metal analysis and it pervades all aspects of sample collection, storage and manipulation. The importance of the problem can be judged by the large number of articles and extensive reviews on the subject which have appeared in recent years (Kosta, 1982; Versieck et al., 1982; Brown et al., 1983; Nieboer and Jusys, 1983; Ihnat, 1988; Versieck, 1983; Versieck, 1984; Schmitt, 1987; Versieck and Cornelis, 1989). It is clear that a great deal of earlier work concerning trace metal analysis of biological tissues and fluids is very unreliable and should be disregarded. This has partly come about with the introduction of more sensitive analytical techniques and their applications to the determination of those metals present in ultra trace quantities. There has thus been a marked downward adjustment of so-called 'normal' or reference ranges for several important metals such as chromium, manganese, nickel and vanadium (Versieck and Cornelis, 1980); Nieboer and Jusys, 1983; Versieck, 1984; Versieck et al., 1988a; Simonoff et al., 1984). It is quite remarkable that until quite recently, mean reference values for chromium in human blood have ranged from 0.14 μg/L to 782 μg/L (Versieck, 1984). A similar problem exists in the case of tissue samples, particularly those obtained by needle biopsy (Versieck, 1983). Problems of contamination can be no less of a problem for metals present in much higher concentration such as zinc (Saleh et al., 1981; Gervin et al., 1983). Some of the major sources of sample contamination have been extensively reviewed by Nieboer and Jusys (1983) and include: air, industrial environment, laboratory construction materials and surfaces, reagents, syringes, sample containers, laboratory wares and tools, etc. Measures to be taken in order to remove or reduce contamination during sample collection or analysis have been reviewed by Zief and Mitchell (1976), Nieboer and Jusys (1983), and Versieck and Cornelis (1989). Some of these measures are quite simple and should be carefully studied before any new investigation is carried out. More elaborate procedures such as the use of clean air or laminar flow installations may be difficult to achieve for all but the specialist laboratory. However, in assigning values for ultra trace metals in certified reference materials they are probably indispens-

able (Nieboer and Jusys, 1983). Major problems remain in controlling contamination in the routine collection of specimens of blood and urine for clinical toxicological and food analysis. Problems can occur frequently in the analysis of many common elements including lead, cadmium, zinc, cadmium, aluminium, manganese and chromium. Laboratories should always specify the use of appropriate specimen collection techniques and containers. Determination for blood lead concentrations in individuals with relatively low blood lead concentrations can be easily invalidated by collection in contaminated blood collection tubes (Crick and Flegal, 1992). Contamination may also occur during a digestion process due to unforeseen contamination of acids or other chemicals.

Speciation

Some of the difficulties in the unbiased determination of certain trace elements in biological materials may be due to problems of speciation. The range of complex organo-metallic species that can be found in nature is very wide (Frausto da Silva and Williams, 1991). In carrying out an analysis for a particular element in any type of biological fluid or tissues, major assumptions are made concerning the precise chemical composition of element species present. Different analytical techniques will have different sensitivities towards particular element species. Much of the early understanding of the special analytical problems posed by element speciation comes from studies of arsenic (Buchet et al., 1980; Buchet et al., 1981) and mercury (Clarkson, 1983). Problems with other metals remain to be resolved and may require considerable analytical sophistication such as in the analysis of chromium speciation (Urasa and Nam, 1989).

Storage

The problem of sample storage is a somewhat neglected area and relatively little is known about the changes that may take place with specimens or reference materials during storage, particularly over many months or years. The problem has been reviewed by Moody (1983) and Stoeppler (1983), who have highlighted some of the potential problems of specimen storage. It is obvious that both specimens and reference materials will undergo some changes during prolonged storage, which will depend upon the type of matrix, storage temperature, the ambient conditions and storage time. Recent studies have shown that some reference materials may have excellent stability characteristics over many years (Braithwaite and Girling, 1988; Zeisler et al., 1988; Dahl et al., 1990). However, the matrix may cause different losses for different elements. At room temperature or 4°C, there will be a progressive deterioration in the biological matrix which may cause important changes in metal speciation or loss of metal by adsorption onto the walls of containers. A volatile metal such as elemental mercury, may be easily lost when the material is reconstituted, but this may depend on the nature of the matrix (Dahl et al., 1990). On the other hand, there may be an increase in metal content due to a gradual contamination from the container material or stopper. This can be a major problem for metals such as aluminium when samples are stored in glass containers (Guillard et al.,

1982; Leung and Henderson, 1983). The use of ultra low temperature storage conditions is obviously one way of minimising changes in specimens and is a useful techniques for the establishment of sample and reference material banks (Moody, 1983; Stoeppler, 1983), but too expensive for routine use. The use of lyophilised standard reference materials can be helpful in avoiding some of the problems of storage of "fresh" material; however, new errors or problems may be introduced when the material is reconstituted.

Personal factors and staff rotation

This is a complex problem, but it is obvious from a number of interlaboratory surveys that despite similar types of methodology being used, widely different findings for laboratory proficiency have been obtained. The conclusion that can be drawn from this observation is that the ability and training of laboratory staff carrying out trace metal determination are important factors which greatly influence the quality of work done. In routine laboratories where a rotation of different staff may be involved in carrying out a certain type of measurement, much attention should be paid to proper training and good laboratory practice, including the use of well documented internal control procedures to try and minimise possible errors. The use of more automated procedures may be one way of reducing the influence of some errors due to variation in human performance. However, the choice of analyst may be more important that the choice of method. Such human factors should be taken into account in the wider aspects of quality assurance and laboratory accreditation.

INTERLABORATORY SURVEYS AND COLLABORATIVE STUDIES

There is quite a long history of carrying out interlaboratory surveys for comparison of findings in respect of precision and accuracy for trace metal analysis of biological samples. Moreover, there are now a number of local, national and international quality assurance schemes in operation which are designed to give retrospective information on the relative performance of laboratories for trace metal analyses. One disadvantage of these schemes is that they are generally not carried out using well characterised pools or certified reference materials, or with reference to results obtained using a definitive method. However, the costs would be prohibitive if a certified reference material were commonly utilized in the operation of a quality assessment scheme. Calculation of the "group" or consensus mean value may have very little relevance to the "true" value in interlaboratory comparison studies carried out by small numbers of laboratories using imprecise or poorly defined methods. However, in large and well organised quality assessment schemes, the group mean (with its dispersion) is usually a good estimate of the true value and the result can be useful for individual accuracy control, or for comparing the precision and bias of different analytical techniques. Most of the early interlaboratory surveys carried out during the 1960's and early 1970's indicated that the reliability of many laboratories carrying out trace metal analyses was very poor. One of the most widely reported early studies was that of Keppler et al. (1970). This set out to evaluate the

performance of laboratories in the USA for carrying out blood lead determinations. It found that only 43% of laboratories obtained similar results (defined as within ± 100 μg/L) for the replicate analysis of a blood specimen containing 650 μg/L lead. The range of reported values for blood lead concentration in this specimen ranged between 0 and 56000 μg/L! Even following the exclusion of two "outliers" the range in reported values was between 300 and 1200 μg/L, spanning the range between 'normal' concentrations and those likely to cause serious toxicity. Several early interlaboratory comparison studies were carried out under the sponsorship of the Commission of the European Communities (Berlin et al., 1973) which reported that the performance of participating laboratories was extremely poor. A later and more detailed European interlaboratory comparison study was carried out by Lauwerys et al. (1975). In this study the performance of 66 participating laboratories was evaluated for the determination of lead, mercury and cadmium in blood, urine and aqueous solutions. Steps were taken by the organisers to ensure homogeneity of sampling: the protocol was designed to try and evaluate the influence of precision, experience and choice of analytical method. However, the results showed up major problems of systematic error between laboratories, even between those demonstrating good precision. The mean interlaboratory relative standard deviation for the determination of three blood lead specimens ranged between 43% and 78% (Lauwerys et al., 1975).

An interesting interlaboratory comparison study for blood lead determination was carried out by Boone et al. (1979). The results reported by 113 participants in a blood lead proficiency testing program organised by the US Centers for Disease Control were compared with results obtained using a definitive method for blood lead analysis – isotope dilution mass-spectrometry (IDMS). The results of this investigation indicated that most of the methods in routine use by laboratories tended to <u>overestimate</u> the actual lead concentration when the 'true' value was less than 400 μg/L and to <u>underestimate</u> the 'true' value at lead concentrations greater than 500 μg/L. Interlaboratory relative standard deviations (according to method) ranged between 29% and 73% for a 'true' lead concentration of 1020 μg/L. It was concluded that all the current methods for blood lead analysis in common use in laboratories showed varying degrees of bias when compared with the results obtained with the definitive method and that the mean value of all the methods' results was not necessarily a reliable estimate of the actual lead content of the sample.

An interesting interlaboratory comparison study concerning the analysis of lead in children's teeth has been reported by Stack and Delves (1982). In this study, representative portions of a single homogeneous tooth powder were sent for lead analysis to thirteen laboratories in six countries that were actively concerned with the analysis of lead concentrations in teeth. An almost 4-fold variation in mean tooth lead concentration was reported by laboratories (range 4.3–15.5 mg/kg). As pointed out by the authors, this range in apparent tooth lead concentrations is approximately the same order of magnitude that would be found for low, medium and high environmental exposure to lead. It would, therefore, seem impossible to compare such data reported by different laboratories, which casts some doubt on the validity of tooth lead measurements as a reliable index of lead exposure in children (Stack and Delves, 1982).

In a more recent evaluation of the bias and reliability of blood lead measurements suing anodic stripping voltammetry (ASV) techniques, interlaboratory comparison showed clear evidence of serious bias (Roda et al., 1988). Use of operating conditions recommended by the manufacturer consistently underestimated blood lead concentrations below 400 μg/L and overestimated blood lead values above 400 μg/L. Lower than expected values were obtained in specimens containing increased concentrations of copper. Modification of the manufacturer's recommended procedure was able to improve the performance of this technique as judged by the analysis of calibration materials previously assayed by isotope dilution-mass spectrometry (Roda et al., 1988). In a subsequent study, quality control data from three different methods of blood lead analysis were evaluated (Osterlow et al., 1990). The three methods evaluated were: anodic stripping voltammetry by a commercial method, a modified voltammetric method, and graphite furnace atomic absorption spectrometry (GFAAS). The methods were compared for precision at a relatively low blood lead concentrations (250 μg/L). Both duplicate and interassay precision were superior for the GFAAS method compared with both voltammetric methods, and significant methodological bias was apparent, although somewhat improved by the modified ASV procedure (Osterloh et al., 1990).

About a decade ago the International Union of Pure and Applied Chemistry (IUPAC) Commission on Toxicology instituted a series of interlaboratory collaborative studies and analytical reviews aimed at harmonizing the analysis of trace metals in biological fluids, which have been extremely useful for a number of important elements e.g. nickel (Sunderman et al., 1982), cadmium (Herber et al., 1990a, 1990b), manganese (Ottaway and Halls, 1986), chromium (Ottaway and Fell, 1986) and selenium (Ihnat et al., 1986b; Welz et al., 1987). These collaborating studies have been extremely wide ranging and have included (a) co-operation in interlaboratory surveys of analytical proficiency; (b) development of reference methods: (c) collaborative interlaboratory trials of reference methods; (d) co-operative assessment of reference materials. All of these IUPAC collaborative studies have had a beneficial influence on work carried out by other groups and organisations concerned with trace element analysis.

As part of the European Communities' programme for investigating environmental lead pollution, extensive surveys covering the nine member states were carried out, involving more than 17,000 subjects (Berlin et al., 1983). During these investigations, special attention was paid to problems of quality assurance and to the task of ensuring compatibility between laboratories. Moreover, biological monitoring of blood lead in the population was delayed for 2 years before it had been established that the large number of participating laboratories could meet the rigid quality control criteria that had previously been agreed. As part of these studies a "reference material" for blood lead analysis was prepared from 'spiked' bovine blood. Although this did not constitute a true reference material which had been given a properly assigned value, it proved invaluable as a common internal quality control material so as to ensure a minimum of bias between laboratories (Berlin et al., 1983). The usefulness of this material was borne out by the final results of the UK part of the EC screening programme (Department of the Environment, 1983). A measure of the validity of the UK data was obtained by exchanging 10% of all the blood lead specimens

with another UK laboratory, also with a Community reference laboratory in Italy. This exercise demonstrated that the agreement between laboratories was very close. In the exchange between the UK and Italian laboratories, 95% of the results were within 20 μg/L, with no significant bias. The agreement between exchanged blood lead specimens provided good evidence of the validity of the monitoring programme and their national and international compatibilities. The use of a common 'reference' or IQC material is essential when planning large international surveys that require a sound basis of accuracy. Vahter and Friberg (1988) have described the use of an extensive quality control procedure as an integral part of a large international exposure monitoring study of lead and cadmium. Both internal and external quality control materials were prepared and sent to participating laboratories. The results showed that good analytical performance for one particular analyte e.g. lead in blood, was no guarantee of good performance with other types of material or at higher or lower concentrations in the same type of material (Vahter and Friberg, 1988).

QUALITY ASSESSMENT SCHEMES

External quality assessment schemes for trace metal analysis are now carried out in many countries, particularly for more important analytes such as lead, cadmium, zinc, copper and aluminium. A national external quality assessment scheme (NEQAS) for blood lead analysis was first established in the UK in 1973 with 37 participating laboratories (Bullock et al., 1986). From its inception, the number of participating laboratories has grown, and at the present time more than 100 laboratories, both in the UK and overseas, participate in the scheme and receive liquid blood lead specimens twice monthly. The material distributed consists of sonicated, anticoagulated human blood from a single human donor which has been spiked with a predetermined amount of lead nitrate. A homogeneous pool of blood is prepared, aliquoted and γ-irradiated prior to distribution to laboratories. Since 1982, cadmium has also been included in the pool (Bullock et al., 1986). During the years of operation, the performance of laboratories has improved significantly; also the types of techniques used for the analysis of blood lead have undergone a major change. Compared with the situation pre-1978, there has been a dramatic improvement in interlaboratory relative standard deviations. It is clear that in the early years of the scheme's operation, performance of most methods and most laboratories was unacceptably poor. During the last 10 years, there has been a marked change towards the use of graphite furnace atomic absorption spectrometry techniques for lead determination and a slow decline in the popularity of other techniques such as the Delves' cup sampling method, although the interlaboratory RSD'S of those using the Delves' technique do not appear to be inferior to the GFTAS methods. Very few laboratories participating in the scheme appear to use voltammetric techniques. Since 1979 the performance of individual laboratories participating in the scheme has been assessed in a continuous fashion using "variance index" parameters (Whitehead, 1977). This involves calculation of both a Variance Index Score (VIS) and a Mean Running Variance Index

Score (MRVIS) which is the average VIS for the 10 most recent returns, the value being updated every time a result is returned by a individual laboratory (Bullock and Wilde, 1985). The continued poor performance of an individual laboratory may have serious consequences quite apart from the clinical implications of such performance. In particular, under current Health and Safety Regulations in the U.K., laboratories whose performance in the NEQAS Scheme for blood lead analysis is poor and remains above a threshold MRVIS score of 80 are not permitted to carry our blood lead assays under the Control of Lead at Work Regulations (Health and Safety Commission, 1986). This MRVIS threshold of 80 corresponds to an average deviation, over 10 specimens, of 12% from the mean (D.G. Bullock, personal communication). It is possible that future UK and European Community legislation concerning the occupational (and environmental) exposure to certain hazardous trace metals may demand a similar external assessment of laboratory performance when carrying out biological monitoring of such metals. Within the WHO and the European Community, there has been an active programme of studies aimed at assessing and improving proficiency in the analysis of a number of trace elements in human biological material, notably lead and cadmium in blood (Vahter, 1982). Data from quality assurance schemes for blood lead analysis in North America in the period 1979-1983 indicated RSD between 10 and 19% at a high value of mean blood lead concentration (50 μg/L) (Saltzman, 1985). A quality control program for the analysis of toxic metals in urine has been carried out in the Nordic countries since 1978 (Valkonen, et al., 1987). More recently, a scheme for the internal and external quality control of blood lead analysis has been organised by the Danish National Institute of Occupational Health using lyophilised human blood (Anglov et al., 1990). A quality assessment scheme for the analysis of a range of trace elements in biological fluids operating from the University of Surrey in the UK has also been described by Taylor and Briggs (1986). This quality assessment scheme involves the preparation of serum, blood and urine samples for a series of 6-monthly cycles. During each cycle 9 different specimens are distributed and each distributed on two separate occasions (Taylor, 1988b). The findings of this scheme has generally shown that experienced laboratories, particularly those specialising in trace element analysis, perform better than inexperienced laboratories or those carrying out measurements infrequently (Taylor, 1988a).

The operation of external quality assessment schemes has been valuable in the case of aluminium analyses. During the last decade there has been a large increase in the number of laboratories carrying out the determination of aluminium in serum, water and dialysis fluids. In the UK there were only about 5 laboratories carrying out such determinations in 1980 but at least 30 were identified 5 years later − a trend that was probably similar in other countries (Taylor, 1990). A number of national and international schemes have been set up in the last decade and have reported remarkably similar findings, (Boyd, 1983; Taylor et al., 1986; Guillard and Pineau, 1986; Stevens et al., 1986; Taylor et al., 1990). In general, laboratory performance for the determination of aluminium in serum is very poor, but even worse in the case of water and dialysate. In the largest scheme which is operated from the University of Surrey, there are 120 participating laboratories in the serum aluminium programme and 55 in the programme for water and dialysis fluid (Taylor,

1990). RSD of 40-60% at 1 μmol/L (27 μg/L) and 20% at 4 μmol/L (108 μg/L) have been commonly observed (Taylor et al., 1986; Taylor, 1990). It is unclear what the reasons are for this poor performance. Methodology of itself does not seem to be a key factor, since most laboratories have been using similar methodology based on graphite furnace atomic absorption spectrometry. The most important factor appears to be the experience of the laboratory carrying out the analysis. For some schemes, there has been a dramatic rise in the number of participants, mostly of laboratories with very little previous experience of trace element analysis (Taylor, 1990). With the exception of a number of experienced laboratories, the performance of a large number of laboratories is insufficient to allow a proper assessment of aluminium exposure (Taylor, 1990). The performance of laboratories has also been impeded by the lack of suitable reference materials, particularly in the case of water and dialysis fluid.

REFERENCE METHODS

It is important to first consider the definition of a "reference" method, as distinct from a "definitive" method of chemical analysis. This problem has been reviewed by Brown (1977) with regard to the development of such techniques for trace metal analysis. It is accepted that a so-called "definitive" method has a negligible bias and is the best approximation to the 'true' value that can be obtained at the point in time. It may also be called the "state of the art" technique. On the other hand, a "reference" method may well be very precise but yet involve some defined bias. In the same context, a "routine" method may suffer from even more bias. However, it is extremely important and useful to try and relate the relative performance of each of these types of methodology, when trying to assign a value to any particular reference material. At the present time, the closest approach to a "definitive" technique is that of stable isotope dilution mass spectrometry: this has been successfully applied to the problem of assigning values to certified reference materials for serum calcium measurement (Brown, 1977). Equally, the availability of such a definitive method for blood lead analysis has in the past been extremely valuable in comparing results obtained by routine techniques (Boone et al., 1979). Stable isotope dilution — mass spectrometric techniques have also been applied to the determination of the very low concentrations of lead that may be found in serum or cerebrospinal fluid (Everson and Patterson, 1980; Everson and Patterson, 1981, Manton and Cook, 1984). These studies have demonstrated that the previously reported values using alternative techniques were gross overestimates of the probable values (Everson and Patterson, 1980; Everson and Patterson, 1981). Further developments of this technique have included the formation of metal chelates which may be subjected to gas chromatography and mass spectrometric analysis (Aggarwal et al., 1990). More recently, inductively coupled plasma emission spectrometry and mass spectrometry (ICP and MS) have become more widely available as powerful techniques for trace metal analysis (Melton et al., 1990; Savory and Wills, 1991; Templeton et al., 1991). These techniques have been applied to the measurement of total lead concentrations and lead isotope ratios in patients' specimens and in refer-

ence materials (Delves and Campbell, 1988). An interesting early example of successful interlaboratory collaboration in the development of a "reference method" was in the determination of total calcium is serum by flame atomic absorption spectrometry. This work has been described in detail by Brown et al. (1981a,b), and it has served as a model for other interlaboratory collaborating studies for the harmonisation of analytical techniques for trace metal analysis. These studies have been successful in the case of nickel, largely due to the development of an internationally approved reference method for the analysis of nickel in serum and urine by graphite furnace atomic absorption spectrometry (Brown et al., 1981c). Improvements in the same technique for the determination of nickel have also undergone continuous development (Sunderman et al., 1986). Graphite furnace atomic absorption spectrometry has continued to be used as a tentative "reference" technique for the determination of a wide range of other trace elements (Delves, 1987; Slavin, 1988). Developments in instrumentation and chemical matrix modification techniques have greatly improved precision of this technique and caused less biased results.

REFERENCE MATERIALS

The inclusion of a certified or surrogate "reference material" in an analytical procedure provides a useful means of quality control and of avoiding gross analytical errors. The roles and applications of standard reference materials have been well described in a useful handbook from the U.S. National Institute of Standards and Technology (Taylor, 1985). It is likely that if more reference materials had been available in earlier years, there would have been fewer discrepancies between laboratories performing trace element analyses. However, the process of assigning reference values for such materials, although very desirable, can be far from simple (Versieck, 1984; Versieck et al., 1988b). There still remains a dearth of suitable reference materials for the routine clinical analysis of some trace elements. During the last 25 years a number of important first and second generation reference materials have become available. Some of these were prepared by early pioneers of the subject, such as Bowen (1967) who developed reference materials of plant origin, such as the well known Bowen's kale. Subsequent interest by agencies such as the U.S. National Institute of Standards and Technology (NIST) saw the early development of a range of first generation biological reference materials derived from bovine liver, orchard leaves, spinach, wheat, rice and brewers yeast. A limited range of reference materials suitable for clinical analysis was subsequently developed. These included a bovine serum reference material for a wide range of major and minor trace elements (NBS- 8419; Veillon et al., 1985) and a reference material (SRM 955) consisting of four levels of lead in porcine blood. However, all of these materials have since been withdrawn from later (1990/91) NIST catalogues. A new serum material of human origin (NBS-SRM 909) is still available, but the nominal value of chromium in the reconstituted material is approximately one hundred times higher than the 'normal' value in human serum (Versieck et al., 1988b). It is only recently that a so-called second generation reference material (freeze dried human serum) has been developed by a Belgian group (Versieck, at

al., 1988b). This material has been prepared free of external contamination and provides the best means of controlling the accuracy of low- level trace element analysis in human serum. A new lead in bovine blood material is also under development by NIST. Other agencies such as the International Atomic Energy Authority (IAEA), have developed a number of non-radioactive reference materials for trace element analysis such as dried blood, calcined bone, fish soluble, milk powder and animal muscle, but these are generally unsuitable for clinical purposes. Other national agencies have started to produce some useful materials. The Commission of the European Communities Community Bureau of Reference initiated a programme some 15 years ago for the development of reference materials which included matrix materials suitable for the control of lead and cadmium determination in blood (Commission of the European Communities, 1985). However, as pointed out by Cornelis and Versieck (1983) many, if not most, of these early materials were unsuitable for what is required in human clinical and toxicological work. As discussed by Cornelis and Versieck (1983) the certified concentrations for Mn, Pb, Cd, Cr, As and Hg in NBS bovine liver exceed those in human serum by a factor of between 10 and 2×10^4. Other non-certified constituent elements are also higher by several orders of magnitude. The consensus view about a decade ago was that there was a clear need for the development of reference materials derived from a human matrix, with certified values comparable with those found in human clinical situations, for biological fluids such as serum, blood, and urine. However, this view did not take into account the problem of obtaining suitable homogeneous materials of human origin or the increasing microbiological risks (e.g. HIV) associated with such materials. Further, true certified reference materials are far too expensive for routine use in internal or external bias control.

Attempts at providing relevant reference materials for trace element analysis have only come to partial fruition in the last 5-10 years. An interesting early commercial venture into the production of reference materials for the determination of "heavy" metals in blood and urine was reported by Muller-Wiegand et al., (1983). A "control" blood for metal analysis at two different concentrations each for lead, cadmium, mercury, and a single concentration of chromium and nickel, was prepared from anticoagulated bovine blood. For urine, a synthetic matrix consisting of electrolytes and organic substances was preferred, which was 'spiked' with different concentrations of the same metals together with arsenic, copper, cobalt and thallium. The materials were characterised and assigned values by analyses carried out by the German "Deutsche Gesellschaft fur Arbeitsmedizin". However, no comparisons were made using definitive methods of analysis such as isotope dilution − mass spectrometry. Leung and Henderson (1983) assessed several commercially available "quality control" sera for possible use as a control material for serum aluminium determinations. Several such sera did appear to have appropriate, and therefore, useful concentrations of aluminium present. However, there were problems if reconstituted materials were stored in the original (glass) container. Satisfactory results were obtained if serum was stored in plastic aluminium-free tubes. There was also a pioneering attempt at assessing the suitability of other established quality control serum preparations for possible use as quality control materials for selenium (Ihnat et al., 1986a,b). In this interlaboratory trial under the auspices of the IUPAC Commission on Toxicology, the

objective was to arrive at a consensus value for the concentration of selenium in two commercially-available (Seronorm) lyophilized human sera, based on about 100 analytical results from 18 laboratories using different analytical techniques.

In a subsequent IUPAC sponsored interlaboratory trial, selenium was determined in a range of lyophilized human materials using hydride generation atomic absorption spectrometry (Welz et al., 1987). The results obtained by the 13 laboratories showed excellent agreement with the values established in previous interlaboratory trials by experienced laboratories using a range of independent techniques.

During the last decade there have been important advances in the development of 'surrogate' reference materials for use in internal bias control. The use of specially prepared bovine haemolysate reference materials for the bias control of blood lead analysis was pioneered by a group of specialist trace element laboratories in the UK (Yeoman, 1983). Several batches of material have been prepared over the last decade and the concentration of lead in the materials accurately assigned by a small group of experienced trace element laboratories, as judged by comparison with results obtained by independent analysis using both thermal ionisation isotope dilution mass spectrometry and inductively coupled plasma emission mass spectrometry (Braithwaite and Girling, 1988; Delves and Campbell, 1988). The materials prepared have been found to be stable over several years providing a continuous basis of unbiased results over long periods of time. Bovine blood lead and cadmium reference materials suitable for internal quality control have also been developed by the Commission of the European Communities Community Bureau of Reference (Commission of the European Communities, 1985). However, these materials have been prepared in lyophilized form, which can have several disadvantages (Braithwaite and Girling, 1988). The development of frozen bovine blood reference materials for the quality control of lead, cadmium and mercury analysis has also been described by the U.S. Centers for Disease Control (Cox, 1989; Cox et al., 1989). Diver et al. (1988) have described the use of human protein and albumin solutions as useful sources for reference materials with trace element concentrations above the ranges found in normal human plasma.

The use of internal quality control materials has been shown to be invaluable in the control of blood lead determinations carried out by a group of specialist trace element laboratories in the UK (Taylor, 1988a). Thus the performance of this group of laboratories has been monitored by an intensive quality control programme over the last decade. The introduction of a rigorous internal quality control procedure with all laboratories using the same bovine reference materials has produced outstanding results regarding laboratory performance characteristics in external quality assessment schemes (Taylor, 1988a). A similar approach is being established for the analysis of aluminium in serum (Taylor, 1988b; Taylor, 1990).

A wider range of 'reference' materials for the routine quality control of trace element analysis has become commercially available in the last few years. A number of diagnostic companies specialising in the production of control materials for biochemical laboratories have produced materials for the control of trace element analysis. Some of these materials have poorly assigned values, and the materials are only of use for the control of labora-

tory precision rather than bias. However, some companies notably Nyegaard (Oslo, Norway) have, through the long term support of IUPAC projects involving a large number of specialist trace element laboratories, produced the best current range of lyophilized human blood serum and urine control materials that are available. The participation of laboratories in assigning reference values has largely been carried out 'free of charge', so that well characterised materials are available to laboratories for everyday use at relatively low cost.

CONCLUDING REMARKS

The last thirty years have seen dramatic improvements in the performance of laboratories regarding the determination of important trace elements. This has come about partly as a consequence of advances in instrumental techniques such as isotope dilution mass spectrometry and graphite furnace atomic absorption spectrometry, but mainly because of the introduction of effective quality assurance and quality control procedures (Brown, 1991). Much of the success of quality control procedures has been brought about by the development of reference materials, including those used in the routine control. However much still remains to be done to improve the general quality of analytical performance of many laboratories carrying out trace metal work; this is particularly true of those operating in the clinical field. The production of suitable reference materials and the further development of definitive or reference methods are important for future progress in internal control as well for harmonisation of interlaboratory findings for trace metal analyses. Quality assurance and accreditation of laboratories are also likely in the future to have a considerable impact on the needs of laboratories to improve and maintain good quality control of trace element analysis.

Future trends in trace element analysis will put even greater pressure on the need to provide unbiased determinations. Increasing interest in the role of trace elements in health and disease will provide the stimulus for the better provision of quantitative determinations on which important decisions are made (Centers for Disease Control, 1991; Moukarzel et al., 1992). Increased public awareness and legislation are likely to bring about substantial reductions in the currently 'acceptable' levels of occupational and environmental exposure to some non-essential elements such as lead, cadmium and aluminium. Determinations that are made as part of the assessment of such exposure will need to be both carefully validated and reproducible over many years or decades (Braithwaite and Brown, 1988; Brown, 1991), which will have a serious impact on laboratory costs. However, reproducible trace element determination with a low bias in biological fluids represents the cornerstone of any proper understanding of the role of trace elements in human health and disease.

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Chapter 11

Reference materials for trace element analysis

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GENERAL PRINCIPLES AND DEFINITIONS

The term "trace element" was originally applied as a qualitative descriptor to any element detected by an analytical procedure, but at such a low level that it could not be quantified; hence it would be reported as being present "only as a trace". During the past half century, however, and particularly during the last twenty years, such important advances in trace element analytical chemistry have occurred that, in principle, all the stable elements can now be identified in biological materials in quantifiable amounts. The enormous growth that has occurred in our knowledge of the role of trace elements in biological systems during the last twenty years is due largely, if not exclusively, to the fact that analytical methods have become available for their unbiased quantification.

This does not mean that all the analytical problems have been solved. Far from it. Evidence from various sources shows that the results of trace element analyses, as currently reported by typical laboratories around the world, may be subject to very large errors indeed. For example, the ratios of highest to lowest laboratory mean values for human blood plasma or serum reported by Versieck and Cornelis (1980, see also Versieck, 1985) are 392 (No. of lab. means = 17) for aluminium, 178 (7) for arsenic, 1321 (30) for chromium, 1352 (14) for cobalt 3.2 (36) for copper, 64 (19) for manganese, 7.6 (6) for mercury, 443 (10) for molybdenum, 138 (21) for nickel, 4.5 (19) for selenium, 3.4 (3) for tin, approx. 12.000 for vanadium, and 5.1 (36) for zinc. The authors conclude that many of the disparities between the values reported by different investigators are due to inadequate sampling and sample handling, or to defective analysis.

While not wishing to diminish the importance of sampling and sample handling, the present author believes that discrepancies of these kinds are in many cases due to defective analysis. Evidence supporting this assertion can be found in the results of many intercomparisons organized by the IAEA in recent years (Parr, 1984, 1985) using some of the reference materials described later in this report.

On the basis of such findings, which do not appear to depend strongly on the level of experience of the laboratories concerned, the following trace elements, judging by laboratories participating in the IAEA's intercomparisons are particularly susceptible to large analytical errors, even amounting to an order of magnitude or more, namely: aluminium, arsenic, cadmium, cobalt, chromium, mercury, manganese, molybdenum, nickel, lead, antimony, selenium and vanadium. In addition, there is some evidence that other elements, such as iodine, silicium and tin, though not often reported in the IAEA's intercomparisons, may also be subject to similar errors. These problems are not only confined to the less-commonly determined trace elements. Some of the more-commonly determined trace elements, such as copper, iron and zinc, may also occasionally give rise to difficulties, depending on the matrix, for example, copper in milk powder (De Goeij et al., 1983).

Improved methods of analytical quality assurance, and their consistent application by analysts everywhere, would thus appear to be urgent requirements in trace element research. To this end, all aspects of quality assurance, both internal and external, need careful attention. In this work, reference materials perform an essential function. Indeed quality assurance without reference materials is largely inconceivable.

Quality assurance

Borrowing from the definition of Taylor (1981), quality assurance is taken to encompass the two concepts *quality control* and *quality assessment.* Quality control is the mechanism established to control errors, while quality assessment is the mechanism used to verify that the measurement system is operating within acceptable limits.

A result for which the uncertainty is unknown is worthless because it cannot be used to draw valid conclusions; worse, it is dangerous because it may be misused and lead to false conclusions. Quality assessment is needed to ascertain that the uncertainty of results reported by a laboratory does not exceed well defined limits, and quality control serves to detect unexpected deviations with a minimum delay.

Internal quality assurance is concerned with maintaining **precision** within a single laboratory, where the importance of keeping careful records, based on reference materials and using appropriate control charts is well established. External quality assurance involves reference materials obtained from an outside body, and is a method for testing the **bias** of laboratory results. In practice, internal quality assurance mainly has to do with laboratory precision, whereas external quality assurance mainly has to do with laboratory bias (ISO 5725, 1986).

Reference materials play an essential role in quality assurance and their use has been endorsed strongly by such bodies as the American Chemical Society Committee on

Environmental Improvement (Keith et al., 1983) and by the Medical Society of the Federal Republic of Germany - Bundesärztekammer (Hansert and Stamm, 1980).

Reference material terminology

Unfortunately, there is a confusing lack of uniformity on the use of technical terms in this field (Parr et al., 1987). Terms such as control material, reference material, research material, standard reference material and certified reference material, can all be found in the literature printed by organizations that produce such materials. Much of this confusion could be dissipated if producers would use some of the vocabulary recently adopted by four international standards organizations (International Organization for Standardization, 1984), namely: BIPM (International Bureau of Weights and Measures), IEC (International Electrotechnical Commission), ISO (International Organization for Standardization) and OIM (International Organization of Legal Metrology).

According to this vocabulary, a reference material is a material or substance one or more properties of which are sufficiently well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials. A certified reference material is a reference material one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body.

The ISO Guide 30 (International Organization for Standardization, 1992) from which these definitions are drawn, proceeds further to define the following quantities:

Certified value: For a CRM, the value that appears in the certificate accompanying the material.

Uncertified value: Value of a quantity, included in the certificate of a CRM or otherwise supplied, which is provided for information only but is not certifeid by the producer or the certifying body.

Consensus value (of a given quantity): For a reference material, the value of the quantity obtained by interlaboratory testing, or by agreement between appropriate bodies or experts. (Note- A consensus value could, through appropriate action by a certifying body, become a certified value.)

Uncertainty of a certain value: Estimate attached to a certified value of a quantity which characterizes the range of value within which the "true value" is asserted to lie with a stated level of confidence.

Precision: The closeness of agreement between independent test results obtained under prescribed conditions (ISO 5725, 1986).

Accuracy: The closeness of agreement between a test result and the accepted reference value (ISO 5725, 1986)

Accepted reference value: A value that serves as an agreed-upon reference for comparison and which is derived as:

a) a theoretical or established value, based on scientific principles;

b) an assigned value, based on experimental work of some national or international organization;

TABLE 1

MAJOR SUPPLIERS OF BIOLOGICAL AND ENVIRONMENTAL REFERENCE MATERIALS
(for more information see Cortes Toro et al., 1990)

Abbreviated name	Full name and address
ARC	Dr. J. Kumpulainen Agricultural Research Centre Central Research Laboratory SF-31600 Jokioinen, Finland
BCR	Community Bureau of Reference (BCR) Commission of the European Communities 200 Rue de la Loi B-1049 Brussels, Belgium
BOWEN	Dr. H.J.M. Bowen West Down, West Street Winterborne Kingston Dorset DT11 9AT, United Kingdom
CZIM	Dr. J. Kucera Nuclear Research Institute 25068 Rez (near Prague) Czech Republic
EPA	U.S. Environmental Protection Agency Environmental Monitoring and Support Laboratory Cincinnati, USA
GHENT	Professor Dr. Jacques Versieck Department of Internal Medicine Division of Gastroenterology University Hospital De Pintelaan 185 B-9000 Ghent, Belgium
KL	Kaulson Laboratories Inc. 687-691 Bloomfield Avenue West Caldwell, NJ 07006, USA
NIES	National Institute for Environmental Studies Japan Environment Agency Yatabe-machi, Tsukuba Ibaraki, 305, Japan
NIST	Standard Reference Materials Room 205, Building 202 National Institute of Standards and Technology Gaithersburg, MD 20899, USA
NRCCM	National Research Centre for Certified Reference Materials Han yongzhi, No.7, District 11 Hepingjie, Chaoyangqu, 100013 Beijing Peoples Republic of China
NRCC	National Research Council Canada Division of Chemistry Ottawa, K1A 0R6, Canada

TABLE 1 (continued)

Abbreviated name	Full name and address
NYCO	NYCOMED Pharma AS Diagnostica Division P.O. Box 4284 Torshov N-0401 Oslo 4, Norway

    c) a consensus value, based on collaborative experimental work under the auspices of a scientific or engineering group.
(ISO 5725, 1986)

Traceability: Property of the result of a measurement or the value of a standard whereby it can be related, with a stated uncertainty, to stated references, usually national or international standards, through an unbroken chain of comparisons.

SUPPLIERS OF REFERENCE MATERIALS

In this section, discussion is limited to suppliers of reference materials which are available internationally, i.e. we are concerned here more with external quality assurance than with internal quality assurance. This is not intended to minimize the importance of reference materials for internal quality assurance; indeed these are essential for monitoring the precision of the analytical methods and for establishing statistical control, e.g. using control charts.

Every analytical laboratory should have its own reference materials for internal quality assurance, and such materials should conform to the same standards of appropriateness, homogeneity and long term stability as are required for certified reference materials (see section "preparation of reference materials). In practice, however, most analytical laboratories do not use their own "in-house" reference materials for internal quality assurance but rather rely on internationally available reference materials. In the opinion of the present author, this is undesirable since much larger amounts are required for internal quality assurance than for external quality assurance, and thereby the available stocks of expensively prepared certified reference materials will be consumed much too quickly.

The major suppliers of internationally available biological reference materials are listed in Table 1 and an overview of their products including type and matrix is presented in Table 2. This information is taken from a (revised) survey by Cortes Toro et al. (1990) and from additonal recent information (Trahey, 1992; Chai Chifang, 1993) which also contain further details pertaining to the elemental concentrations and their uncertainties, the unit weight or volume of material supplied, its cost, and the minumum weight of material recommended for analysis. Another revision of the survey by Cortes Toro et al. (1990) is at present in preparation and will appear soon (Parr et al., 1994).

TABLE 2

OVERVIEW OF BIOLOGICAL REFERENCE MATERIALS AND ELEMENTS QUOTED FOR THE SUPPLIERS LISTED IN TABLE 1.

If the element symbol is underlined, this indicates that a certified or recommended value is available; if not underlined, only an information value is available. Because of frequent changes in available and the offer of new materials the reader should consult actual catalogues of suppliers or other information (e.g. Klich and Walker, 1993)

Material	Code No.	Quoted elements

Agricultural Research Centre Jokioinen, Finland (ARC)

Animal Muscle (pork)	ARC/CL-AM	Ca Cd Cu Fe Mg Mn Mo Pb Se Zn
Milk Powder	ARC/CL-MP	Ca Cd Cu Fe Hg Mg Mn Mo Pb Se Zn
Potato Powder	ARC/CL-PP	Ca Cd Cu Fe Mg Mn Mo Ni Pb Se Zn
Total Diet	ARC/CL-TD	Ca Cd Cu Fe Hg K Mg Mn Mo Na Ni Pb Se Zn
Wheat Flour	ARC/CL-WF	Ca Cd Cu Fe Hg Mg Mn Mo Ni Pb Se Zn

Community Bureau of Reference (BCR), CEC (BCR, 1992)

Aquatic Plant	BCR-CRM-060	Al Ca Cd Cl Cu Fe Hg K Mg Mn N Na P Pb S Si Ti Zn
Aquatic Plant	BCR-CRM-061	Al Ca Cd Cl Cu Fe Hg K Mg Mn N Na P Pb S Si Ti Zn
Olive Leaves	BCR-CRM-062	Al Ca Cd Cl Cu Fe Hg K Mg Mn N Na P Pb S Si Ti Zn
Spruce Needles	BCR-CRM-101	Al C Ca Cd Cl Cr Cu Fe H K Mg Mn Mo N P Pb S Zn
Skim Milk Powder	BCR-CRM-063	Ca Cd Cl Co Cu Fe Hg K Mg Mn N Na Ni P Pb Se Tl Zn
Skim Milk Powder (Lower level spiked)	BCR-CRM-150	Cd Co Cu Fe Hg I Mn Ni Pb Se Tl Zn
Skim Milk Powder (Higher level spiked)	BCR-CRM-151	Cd Co Cu Fe Hg I Mn Ni Pb Se Tl Zn
Bovine Muscle	BCR-CRM-184	As Ca Cd Cl Cr Cu Fe Hg I K Mg Mn Na Ni P Pb Se Zn
Pig Kidney	BCR-CRM-186	As Ca Cd Cl Cr Cu Fe Hg I K Mg Mn Na Ni P Pb Se Zn
Wholemeal Flour	BCR-CRM-189	As Ca Cd Cl Cr Cu Fe Hg K Mg Mn Na Ni Pb Se Zn
Brown Bread	BCR-CRM-191	As Ca Cd Cl Cr Cu Fe Hg K Mg Mn Na Ni Pb Zn
Bovine Blood (Natural level)	BCR-CRM-194	Cd Pb
Bovine Blood (Lower level spiked)	BCR-CRM-195	Cd Pb

TABLE 2 (continued)

Material	Code No.	Quoted elements
Bovine Blood (Higher level spiked)	BCR-CRM-196	Cd Pb
Single Cell Protein	BCR-CRM 273	Ca Fe K Mg N P
Single Cell Protein	BCR-CRM-274	As Cd Co Cu F I Mn Ni Pb Se Zn
Mussel Tissue	BCR-CRM-278	As Cd Cr Cu Fe Hg Mn Pb Se Zn
Sea Lettuce	BCR-CRM-279	As Br C Cd Cr Cu Fe Hg I K N Pb Se Zn
Rye Grass	BCR-CRM-281	As B Cd Cr Co Cu Fe Hg K Mg Mn Mo N P Sb Zn
Human Hair	BCR-CRM-397	As Cd Cu Hg P Pb Se Zn
Plankton	BCR-CRM-414	As Cd Cr Co Cu Fe Hg K Mn Mo Ni Pb Sc Se Sr V Zn
Cod Muscle	BCR-CRM-422	As Br Ca Cd Co Cu Fe Hg I K Mg Mn Pb S Se Sr Zn

Bowen

Kale	BOWEN's Kale	Ag Al As Au B Ba Br C Ca Cd Ce Cl Co Cr Cs Cu Eu Fe Ga H Hf Hg I In K La Li Lu Mg Mn Mo N Na Ni O P Pb Rb Ru S S

Nuclear Reasearch Institute Rez near Prague, Czech Republic (CZIM)

Bovine Liver	CZIM-LIVER	Ag As Br Ca Cd Cl Co Cr Cs Cu Fe Hg K La Mg Mn Mo Na Pb Rb S Sb Se Sn Sr V Zn

U.S. Environmental Protection Agency (EPA)

Fish	EPA-FISH	As Cd Cr Cu Hg Ni Pb Se Zn

Versieck (GHENT)

Human Serum	GHENT SERUM	Al As Br Cd Co Cr Cs Cu Fe Mn Mo Rb Se Zn

International Atomic Energy Agency (IAEA)

Milk Powder	IAEA-A-11	As Ca Cd Cl Co Cr Cu Fe Hg I K Mg Mn Mo Na Ni P Pb Rb Se Zn
Animal Blood	IAEA-A-13	Br Ca Cu Fe K Mg Na Ni P Pb Rb S Se Zn
Animal Bone	IAEA-H-5	Ba Br Ca Cl Fe K Mg Na P Pb Sr Zn
Copepoda	IAEA-MA-A-1/TM	Ag As Cd Co Cr Cu Fe Hg Mn Ni Pb Sb Se Zn
Fish Flesh	IAEA-MA-A-2/TM	Ag As Cd Co Cr Cu Fe Hg Mn Ni Pb Sb Se Zn
Rye Flour	IAEA-V-8	Al Au Ba Br Ca Cd Cl Co Cs Cu Fe K Mg Mn Mo Na P Rb S Sb Zn

TABLE 2 (continued)

Material	Code No.	Quoted elements
Cotton Cellulose	IAEA-V-9	Al <u>Ba</u> Br <u>Ca</u> Cd <u>Cl</u> <u>Cr</u> <u>Cu</u> Fe Ga Hf <u>Hg</u> Li <u>Mg</u> <u>Mn</u> <u>Mo</u> Na <u>Ni</u> <u>Pb</u> S Sc Se Sm Sn <u>Sr</u> Th U V
Hay Powder	IAEA-V-10	Al <u>Ba</u> Br <u>Ca</u> <u>Cd</u> Co <u>Cr</u> Cs <u>Cu</u> Eu <u>Fe</u> <u>Hg</u> K La <u>Mg</u> Mn <u>Mo</u> Na <u>Ni</u> P <u>Rb</u> Sb <u>Sc</u> Se <u>Sr</u> Zn
Milk Powder	IAEA-153	<u>Br</u> <u>Ca</u> <u>Fe</u> <u>K</u> <u>Mg</u> <u>Na</u> <u>P</u> <u>Rb</u> <u>Zn</u>

Kaulson Laboratories Inc., USA (KL)

Material	Code No.	Quoted elements
Blood (Lead level high)	KL-100-H	<u>Pb</u>
Blood (Lead level medium)	KL-100-M	<u>Pb</u>
Blood (Lead level low)	KL-100-L	<u>Pb</u>
Urine (Lead level -too- high)	KL-110-H	<u>Pb</u>
Urine (Lead level medium)	KL-110-M	<u>Pb</u>
Urine (Lead level low)	KL-110-L	<u>Pb</u>
Urine (Lower level)	KL-140-I	<u>As</u> <u>Cd</u> <u>Hg</u>
Urine (Higher level)	KL-140-II	<u>As</u> <u>Cd</u> <u>Hg</u>
Urine (High level)	KL-142-I	<u>Be</u> <u>Cr</u> <u>Ni</u> <u>Se</u>
Urine (lower level)	KL-142-II	<u>Be</u> <u>Cr</u> <u>Ni</u> <u>Se</u>
Serum (Lower for Cu and Fe)	KL-146-I	<u>Cu</u> <u>Fe</u> <u>Zn</u>
Serum (Higher for Cu and Fe)	KL-146-II	<u>Cu</u> <u>Fe</u> <u>Zn</u>
Serum (Lower for Al and Mn)	KL-147-I	<u>Al</u> <u>Mg</u> <u>Mn</u>
Serum (Higher for Al and Mn)	KL-147-II	<u>Al</u> <u>Mg</u> <u>Mn</u>
Serum (Lower level)	KI-148-I	<u>Be</u> <u>Cr</u> <u>Ni</u> <u>Se</u>
Serum (Higher level)	KL-148-II	<u>Be</u> <u>Cr</u> <u>Ni</u> <u>Se</u>

National Institute for Environmental Studies, Japan (NIES)

Material	Code No.	Quoted elements
Chlorella	NIES-CRM-3	<u>Ca</u> Cd <u>Co</u> <u>Cu</u> <u>Fe</u> <u>K</u> <u>Mg</u> <u>Mn</u> P Pb Sc <u>Sr</u> <u>Zn</u>
Tea Leaves	NIES-CRM-7	<u>Al</u> Ba <u>Ca</u> <u>Cd</u> Co Cr Cs <u>Cu</u> <u>K</u> <u>Mg</u> <u>Mn</u> <u>Pb</u> Sb Sc Sr <u>Zn</u>

TABLE 2 (continued)

Material	Code No.	Quoted elements
Sargasso	NiES-CRM-9	Ag Al As Br Ca Cd Cl Co Cr Cs Cu Fe Mg Mn Na P Pb Rb S Sb Sc Se Sr Ti U V Zn
Rice Flour (Cd content low)	NIES-CRM-10A	Al As Br Ca Cd Cl Co Cr Cu Fe Hg K Mg Mn Mo Na Ni P Rb Se Sr Zn
Rice Flour (Cd content low)	NIES-CRM-10B	Al As Br Ca Cd Cl Co Cr Cu Fe Hg K Mg Mn Mo Na Ni P Rb Se Sr Zn
Rice Flour (Cd content low)	NIES-CRM-10C	Al As Br Ca Cd Cl Co Cr Cu Fe Hg K Mg Mn Mo Na Ni P Rb Se Sr Zn

National Institute of Standards and Technology (NIST) (Trahey, 1992)

Apple Leaves	NIST-SRM-1515	Al As B Ba Br Ca Cd Ce Cl Co Cr Cu Eu Fe Hg I K La Mg Mn Mo N Na Ni Pb Rb S Sb Sc Se Sm Sn Sr Tb Th U V Zn
Peach Leaves	NIST-SRM-1547	Al As B Ba Br Ca Cd Ce Cl Co Cr Cu Eu Fe Hg I K La Mg Mn Mo N Na Ni Pb S Sb Sc Se Sm Sn Sr Tb Th U V Zn
Total Diet	NIST-SRM-1548	Al Ca Cd Cl Cu Fe K Mg Mn Mo N Na Ni P Pb Rb S Se Sn Zn
Non-fat Milk Powder	NIST-SRM-1549	Ag Al As Br Ca Cd Cl Co Cr Cu F Fe Hg I K Mg Mn Mo Na P Pb S Sb Se Zn
Oyster Tissue	NIST-SRM-1566a	Ag Al As Ca Cd Cl Co Cr Cu F Fe Hg I K Mg Mn Ni Na Ni P Pb Rb S Sb Se Sn Sr Th U V Zn
Wheat Flour	NIST-SRM-1567a	Al As Br Ca Cd Co Cu Fe Hg I K Mg Mn Mo Na P Pb Rb S Se Sn U V Zn
Rice Flour	NIST-SRM-1568a	Al As Br Ca Cd Cl Co Cu Fe Hg I K Mg Mn Mo Na Ni P Pb Rb S Sb Se Sn Te U V Zn
Brewers Yeast	NIST-SRM-1569	Cr
Pine Needles	NIST-SRM-1575	Al As Br Ca Cd Ce Co Cr Cu Eu Fe Mn N Ni P Pb Rb Sb Sc Sc Th Tl U
Bovine Liver	NIST-SRM-1577b	Ag Al As Br Ca Cd Cl Co Cu Fe Hg K Mg Mn Mo N Na P Rb S Sb Se Sr Tl U V Zn
Lead in Blood	NIST-SRM-955a	in preparation
Freeze dried Urine Toxic Metals (set of two normal and elevated level)	NIST-SRM 2670	Al As/As Au Be Ca Cd/Cd Cl Cr/Cr Cu Hg/Hg K Mg Mn Na Ni Pb/Pb Pt Se SO$_4$ V
Freeze dried Urine (normal and elevated level)	NIST-SRM-2672a	Hg/Hg
Corn Stalk	NIST-RM-8412	Ca Cl Cu F Fe K Mg Mn N Na Se Sr Zn
Corn Cernel	NIST-RM-8413	Al Ca Cl Cu F Fe K Mg Mn N Se Zn

USA/Canada Collaborative Materials (powder form) - For details see 1992/93 Cata-

TABLE 2 (continued)

Material	Code No.	Quoted elements
Whole Egg Powder	RM-8415	23 Elements certified
Microcryst. Cellulose	RM-8416	8 Elements certified
Wheat Gluten	RM-8418	24 Elements certified
Corn Starch	RM-8432	12 Elements certified
Corn Bran	RM-8433	27 Elements certified
Whole Milk Powder	RM-8435	20 Elements certified
Durum Wheat Flour	RM-8436	25 Elements certified
Hard red Spring Wheat Flour	RM-8437	15 Elements certified
Soft Winter Wheat Flour	RM-8438	14 Elements certified

National Research Centre for Certified Reference Materials (GBW)

Human Hair	GBW-09101	Ag Al As Ba Br Ca Cd Cl Co Cr Cu Fe Hg I K La Mg Mn Mo Na Ni P Pb S Sb Sc Sc Sr V Zn

Other biological reference materials (overview, for details see Chai Chifang, 1993):

Human Hair	GBW-07601	32 Elements certified, 7 noncertified
Shrub Leaves	GBW-07602	42 Elements certified, 7 noncertified
Poplar Leaves	GBW-07604	41 Elements certifeid, 8 noncertified
Tea	GBW-07605	37 Elements certified, 15 noncertified
Peach Leaves	GBW-08501	13 Elements certified, 3 noncertified
Rice Flour	GBW-08502	12 Elements certified
Wheat Flour	GBW-08503	10 Elements certified, 4 noncertified
Cabbage	GBW-08504	15 Elements certified, 1 noncertified
Tea	GBW-08505	22 Elements certified, 8 noncertified
Pork Liver	GBW-08551	15 Elements certified, 3 noncertified
Mussel	GBW-08571	16 Elements certified, 4 noncertified
Freeze Dried Urine	GBW-09103	10 Elements certified
Bovine Serum	GBW-09131	8 Elements certified
Codonopsis Pilosuna	GBW-09501	7 Elements certified, 3 noncertified

National Research Council Canada (NRCC)

Dogfish Liver	NRCC-DOLT-1	As Cd Cl Co Cr Cu Fe Hg Methyl-Hg K Mg Mn Na Ni Pb Se Zn
Dogfish Muscle	NRCC-DORM-1	As Cd Cl Co Cr Cu Fe Hg Methyl-Hg K Mg Mn Na Ni Pb Se Zn
Lobster Hepatopancreas	NRCC-TORT-1	As Ca Cd Cl Co Cr Cu Fe Hg Methyl-Hg K Mg Mn Mo Na Ni P Pb S Se Sn Sr V Zn
Non Defatted Lobster Hepatopancreas	NRCC-LUTS-1	Ag As Ca Cd Cr Co Cu Fe Hg Methyl-Hg K Mg Mn Ni Pb Se Sr Zn

TABLE 2 (continued)

Material	Code No.	Quoted elements
Nycomed Pharma AS (STE) (Thomassen, personal communication,1993)		
Human Serum	STE-10017	Ca Cu Fe Hg K Mg Na Ni Pb Se Zn
HumanUrine	STE-9021	Al As Ca Cd Co Cr Cu F Fe Hg K Mg Mn Na Ni Pb Se Tl Zn
Human Urine	STE-9024	see STE 9021
Human Whole Blood (Low level)	STE-052	Cd Hg Pb Se
Human Whole Blood (Medium level)	STE-053	Cd Hg Pb Se
Human Whole Blood (Higher level)	STE-056	Cd Hg Pb Se

PREPARATION OF REFERENCE MATERIALS

Selection of starting material

One of the guiding principles behind all trace analysis work is that contamination should be reduced to the level of insignificance. The same principle should also be applied in the preparation of reference materials, even though a small level of contamination can, in practice be tolerated, provided that it is uniformly distributed. Unfortunately, however, the preparation of large batches of reference materials under conditions that guarantee minimal contamination is a difficult and expensive proposition. For this reason, at the IAEA, it has generally been necessary to use as starting material some industrial product (e.g. rye flour, milk powder, or animal bone powder) originally prepared in powdered form for purely commercial purposes. Obviously, for such materials, it is not always possible to guarantee that contamination is insignificant for all elements of interest. Contamination with cobalt and chromium, for example, arising from the use of containers or tools made of stainless steel, is almost to be expected. This has certainly been a problem with some of the IAEA's reference materials such as H-5 animal bone, and also, as pointed out by Dams (1983), has not been entirely avoided in some of the earlier NIST materials such as SRM-1577 bovine liver and SRM-1569 brewers yeast.

By avoiding contamination, one can in principle come close to the ideal goal of producing an appropriate reference material, i.e. one that not only has the same matrix as that of the samples to be analysed but also matches them with respect to the levels of the trace elements of interest. As is also pointed out by Dams (1983) it is desirable that speciation (i.e. the valency and chemical binding of trace elements) should be the same as in the real matrix. In other words, reference materials should preferably be made of natural products with a similar matrix to that of the samples to be analysed, and not out of

a mixture of chemicals put together in the laboratory. This is important not only for speciation studies as such, but also because the analytical method may be affected by the chemical species even when interest is confined to a determination of the total concentration of the trace element. Such problems, for example, have been documented in the case of chromium by de Goeij et al. (1978). However, despite a widespread interest that exists in speciation studies only very few reference materials are available or in preparation at present for specific trace element-species.

A further important requirement in selecting the starting material is that, after all the processing has been done to produce the end product, a large amount should remain to permit meaningful use of this material over a period of several years. Most producers do not provide details of how much is available for distribution. The IAEA, for its part, has adopted the criterion that, for a trace element reference material, at least 50 kg of the final end product should be produced. In practice, however, some of the reference materials already issued by the IAEA have been produced in amounts as small as 15 kg, and sometimes less (for human hair, HH-1, only 300 g was produced, with the consequence that stocks were exhausted very quickly).

Homogenization and testing

One of the critical steps in preparing a reference material is homogenization. Generally, this is accomplished using commercially available ball or disc mills (with the consequent risk of contamination). Some special procedures have also been developed, such as the use of a disc mill operated at cryogenic temperatures modelled on a design by Zeisler et al. (1983). Another approach, used for fresh materials of the German Environmental Specimen Bank and for larger batches is a cryogenic grinding system using either stainless steel, PTFE or titanium rods and grinding cylinders (Schladot and Backhaus, 1988, Schladot et al., 1992). The first system was used for the preparation of IAEA mixed human diet, H-9, the second very recently for the homogenization of a batch of human hair in a preliminary study to develop a new hair reference material. The process of homogenization can also be assisted by passing the material through a sieve. Nylon sieves with a mesh size of 125 μm are generally used for this purpose at the IAEA.

For practical reasons, homogeneity cannot usually be tested for every element of interest. In practice, at the IAEA, homogeneity is checked by determining the concentrations of one or more major elements (e.g. sodium) and one or more trace elements (e.g. zinc) by INAA of several subsamples from various bottles chosen at random. For homogeneity tests of a number of trace elements also the solid sampling AAS technique can be used with an appropriate number of subsamples weighing around 1 mg or even less from various bottles (Kurfürst et al., 1993). On the basis of such measurements, the homogeneity is usually better than 2% relative standard deviation (RSD) for samples of ≥ 100 mg dry weight.

Statements of the first kind about homogeneity, however, may in future no longer be considered sufficient. The International Standards Organization (1981) is already moving

towards the position that homogeneity should be expressed in terms of the sampling constant, K_s defined by

$$K_s = s^2 m$$

where s is the relative standard deviation in per cent for one component of the sample (i.e. the subsampling uncertainty) and m is the subsample mass. In principle, K_s will have to be defined separately for each element.

Storage and stability

The stability of a reference material is of great importance since the same material may be used over a period of many years. At issue is not only the question of whether it continues to be pleasant to handle (biological materials can of course be attacked by bacteria, fungi, insects and other pests), but also that, due to evaporation or chemical reactions, the concentrations and chemical binding of some of the elements of interest may change. This is obviously of greatest concern for elements that can exist in a volatile form such as mercury and arsenic, which could thereby be lost.

Most biological reference materials may be assumed to be relatively stable if stored in the form of a dry powder. A further extension of lifespan can be expected as a result of radiation sterilization, which is commonly applied to IAEA biological reference materials. Finally, the user is well advised to store his reference materials in a refrigerator, or even a deep-freeze cabinet (though this recommendation is usually not stated in the documentation that accompanies the reference material).

According to ISO (1981), the period of validity of the reference material should be stated by the issuing organization. In practice, however, this is usually not done for the simple reason that the producer has no reliable means to determine the lifespan of the product.

CERTIFICATION OF REFERENCE MATERIALS

Although certification is the desirable end goal of the procedure used to establish the composition of a reference material, producers are also concerned with lesser degrees of certainty such as are represented by consensus values, best estimates or even "information values".

Procedures

ISO Guide 35 (1985) recognizes three different measurement approaches, or combinations of these three, used by certifiers. They are:

a) Measurement by a single definitive method in a single laboratory. The method is usually performed by two or more analysts working independently. Frequently, an

accurately characterized back-up method is employed to provide additional assurance that the data are correct.

b)   Measurement by two or more independent reference methods in one laboratory. The methods must have small estimated inaccuracies relative to the end-use certification requirement.

c)   Measurement by a network of qualified laboratories using one or more methods of demonstrated accuracy.

In practice, the first of these approaches cannot be applied to trace elements because there is no agreement yet on the existence of definitive methods of analysis. Reliance therefore has to be placed on the remaining two approaches, of which (b) is the most commonly used by NIST, and (c) is the most commonly used by e.g. BCR and IAEA. With respect to (c) there are also important differences according to whether the network of laboratories is selected by the producer of the reference material (the normal procedure adopted by BCR) or whether the participants are self-selected (the normal procedure adopted by IAEA).

Each of these procedures has its own advantages and disadvantages, but each, if properly applied, is capable of leading to reliable certification. The methods used at IAEA (Parr, 1984, Parr et al. 1988) involve a dual approach. On the one hand, statistical tests are applied to eliminate outliers; in addition, however, various acceptance criteria are applied of which the most important are (1) that data should be available from at least two different analytical methods for the calculation of the consensus value, and (2) that there should be no significant differences between the groups of accepted results obtained by different analytical methods.

Contents of certificates

ISO Guide 31 (1981 b) identifies various kinds of information which should be included in certificates of reference materials, but in practice, biological reference materials for trace element studies rarely, if ever, meet all these criteria. The required information includes: (1) name and address of the certifying organization; (2) title of the document; (3) status of the certificate; (4) name of material; (5) sample number and/or batch number; (6) date of certification; (7) availability of other forms/sizes of the reference materials; (8) source of the reference material; (9) supplier of the reference material; (10) preparer of the reference material; (11) description of the reference material: (12) statement of intended use; (13) stability, transportation and storage instructions; (14) special instructions of correct use; (15) method of preparation; (16) statement of homogeneity; (17) certified property values and their uncertainty; (18) secondary property values given for information but not certified; (19) special values obtained by individual laboratories or methods; (20) meaning of the statistical uncertainty; (21) measurement techniques used for certification; (22) names of analysts, investigators, and participating laboratories; (23) legal notice; (24) reference (including companion report if any); (25) signatures or names of certifying officers.

One of the most important requirements in the present writer's opinion is to reach agreement on how to express the uncertainties in the certified values and to make the meanings of these quantities more comprehensible to the user. ISO Guide 35 (1985) recognizes two different kinds of confidence interval, One is the confidence interval of the mean (usually expressed as the 95% confidence level, taking account of the degrees of freedom implied by the number of measurements). The other is the statistical tolerance interval, which may be applied to materials in which unit-to-unit variation (inhomogeneity) is not negligible compared to the measurement uncertainty. The tolerance interval is usually constructed so that it will cover 95% of the population with a probability of 99%. Both these ways of expressing certified values and their uncertainties are based on the assumption of normal statistics. However, ISO Guide 35 (1985) goes on to recognize that, in the case of very irregular distributions, such as may be found for example in trace element analysis, the use of a more robust statistic such as the median or a trimmed mean my be appropriate. Many of the consensus values reported for IAEA reference materials are, in fact, calculated in this way

SELECTED DATA FOR APPROPRIATE REFERENCE MATERIALS

Examples for reference materials useful for instrument calibration, method validation and development in the field of human materials for which certified or other kinds of concentration values are reported for the 13 trace elements considered in this book (Al, As, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Se, Tl, V and Zn) are given in Table 3. The data are taken from the survey prepared by Cortes Toro et al. (1990) and from other sources (BCR, 1992, Trahey, 1992, Chai Chifang, 1993) which the reader should consult for further details. Most of the columns are self explanatory. Column T contains a code (C = certified, N = noncertified or information value) for the type of reference value specified by the issuing authority. The uncertainty in the concentration value is expressed as a percentage error, but the meaning of this may differ somewhat from one material to another. In most cases it expresses the 95% confidence interval of the mean, but in a few other cases a tolerance interval, or some other definition (sometimes unspecified), may have been used by the producer.

These data are presented in a way that is intended to help the user select a reference material that matches as closely as possible (i.e. with respect to matrix type and concentration of the element of interest) the "real" samples that are to be analyzed.

It will be apparent from these data that, of the elements considered in this book, there is a serious lack of suitable reference materials for aluminium, vanadium and thallium. In addition, the uncertainties in the recommended concentrations are generally excessively large for all the elements considered in this book, except possibly copper, manganese and zinc.

TABLE 3

APPROPRIATE BIOLOGICAL REFERENCE MATERIALS USEFUL FOR INSTRUMENT CALIBRATION, METHOD VALIDATION AND DEVELOPMENT FOR HUMAN MATERIALS AND THE 13 ELEMENTS TREATED IN THIS BOOK

The materials are listed in descending order of the elemental concentration; the latter is expressed on a dry weight basis unless otherwise noted by an asterix in column T (see text for further explanations)

Material	Code	T	Concentration mg/kg or mg/L	Error %
Al: Aluminium				
Human Hair	GBW-09101	C	13.3	25
Non-fat Milk Powder	NIST-SRM-1549	N	2	
Bovine Liver	NIST-SRM-1577b	N	2	
Serum	KL-147-II	C*	0.7	21
Serum	KL-147-I	C*	0.35	17
Urine (Normal)	NIST-SRM-2670	N*	0.18	
Urine (Spiked)	NIST-SRM-2670	N*	0.18	
Urine	STE-9024	N*	0.16	
Urine	STE-9021	N*	0.07	
Human Serum	GHENT-SERUM	C	0.02	
As: Arsenic				
Human Hair	GBW-09101	C	0.59	11
Urine (Spiked)	NIST-SRM-2670	C*	0.48	21
Human Hair	BCR-CRM-397	N	0.31	
Human hair	GBW-07601	C	0.28	18
Urine	STE-9021/24	N*	0.2	
Urine	KL-140-II	C*	0.15	20
Single Cell Protein	BCR-CRM-274	C	0.132	34
Bovine Liver	CZIM-LIVER	N	0.11	
Pig Kidney	BCR-CRM-186	C	0.063	14
Urine	KL-140-I	C*	0.05	25
Bovine Liver	NIST-SRM-1577b	C	0.047	13
Bovine Muscle	BCR-CRM-184	N	0.026	
Urine (Normal)	NIST-SRM-2670	N*	0.015	
Milk Powder	NIST-SRM-1549	N	0.002	

TABLE 3 (continued)

Material	Code	T	Concentration mg/kg or mg/L	Error %
Cd: Cadmium				
Pig Kidney	BCR-CRM-186	C	2.71	5.5
Human Hair	BCR-CRM-397	C	0.521	4.4
Bovine Liver	CZIM-LIVER	C	0.48	6.3
Bovine Liver	NIST-SRM-1577b	C	0.44	14
Human Hair	GBW-07601	C	0.11	27
Human Hair	GBW-09101	C	0.095	13
Urine (Spiked)	NIST-SRM-2670	C*	0.088	3.4
Freeze-dried Urine	GBW-09103	C	0.053	5.6
Single Cell Protein	BCR-CRM-274	C	0.030	6.7
Urine	KL-140-II	C*	0.03	20
Animal Muscle (pork)	ARC/CL-AM	C	0.022	18.6
Whole Blood	STE-056	N*	0.019	
Bovine Muscle	BCR-CRM-184	C	0.013	15
Bovine Blood	BCR-CRM-196	C*	0.0124	4
Whole Blood	STE-053	N*	0.0124	
Urine	KL-140-I	C*	0.01	25
Urine	STE-9021/24	N*	0.0062	
Whole Blood	STE-052	N*	0.0056	
Bovine Blood	BCR-CRM-195	C*	0.0054	4.5
Human Serum	GHENT-SERUM	C	0.0020	20
Bovine Blood	BCR-CRM-194	C*	0.0005	40
Urine (Normal)	NIST-SRM-2670	N*	0.0004	
Cr. Chromium				
Urine	KL-142-I	C*	10	50
Human Hair	GBW-09101	C	4.77	8
Human Hair	GBW-07601	C	0.37	16
Urine	KL-142-II	C*	0.2	37
Serum	KL-148-I	C*	0.15	40
Freeze-dried Urine	GBW-09103	C	0.091	6.6
Urine (Spiked)	NIST-SRM-2670	C*	0.085	7.1
Bovine Muscle	BCR-CRM-184	N	0.076	
Serum	KL-148-I	C*	0.075	80
Pig Kidney	BCR-CRM-186	N	0.058	
Bovine Liver	CZIM-LIVER	N	0.044	
Urine	STE-9021/24	N*	0.022	
Urine (Normal)	NIST-SRM-3670	N*	0.013	
Human Serum	GHENT-SERUM	C	0.00076	13

TABLE 3 (continued)

Material	Code	T	Concentration mg/kg or mg/L	Error %
Cu: Copper				
Human Hair	GBW-09101	C	23	6.1
Human Serum	GHENT-SERUM	C	11.1	3.6
Human Hair	GBW-07601	C	10.6	11
Animal Blood	IAEA-A-13	C	4.3	13
Animal Muscle (pork)	ARC/CI-AM	C	2.68	10.4
Bovine Muscle	BCR-CRM-184	C	2.36	2.5
Serum	KL-146-II	C*	1.5	20
Human Serum	STE-10017	N*	1.1	
SERUM	KL-146-I	C*	0.7	21
Urine (Spiked)	NIST-SRM-2670	C*	0.37	8.1
Urine (Normal)	NIST-SRM-2670	C*	0.13	15
Human Hair	BCR-CRM-397	N	0.11	
Urine	STE-9021/24	N*	0.045	
Hg: Total Mercury				
Human Hair	BCR-CRM-357	C	12.3	4.9
Human Hair	GBW-09101	C	2.16	9.7
Bovine Liver	CZIM-LIVER	C	0.37	5.7
Human hair	GBW-07601	C	0.36	22
Urine (Spiked)	NIST-SRM-2670	C*	0.105	7.6
Urine	KL-140-II	C*	0.06	20
Urine	STE-9021/24	N*	0.051	
Animal Muscle (pork)	ARC/CL-AM	C	0.022	13.6
Urine	KL-140-I	C*	0.02	25
Whole Blood	STE-056	N*	0.014	
Whole Blood	STE-053	N*	0.01	
Whole Blood	STE-052	N*	0.004	
Urine (Normal)	NIST-SRM-2670	N*	0.002	
Human Serum	STE-10017	N*	0.0011	
Mn Manganese				
Bovine Liver	NIST-SRM-1577b	C	9.9	8.1
Pig Kidney	BCR-CRM-186	C	8.5	3.5
Bovine Liver	CZIM-LIVER	C	7.6	6.6
Human Hair	GBW-07601	C	6.3	12.6
Human Hair	GBW-09101	C	2.94	6.8
Bovine Muscle	BCR-CRM-184	C	0.334	8.4

TABLE 3 (continued)

Material	Code	T	Concentration mg/kg or mg/L	Error %
Urine (Spiked)	NIST-SRM-2670	N*	0.33	
Animal Muscle (pork)	ARC/CL-AM	C	0.30	8
Freeze-dried Urine	GBW-09103	C	0.29	10
Serum	KL-147-II	C*	0.04	37
Serum	KL-147-I	C*	0.03	33
Urine (Normal)	NIST-SRM-2670	N*	0.03	
Urine	STE-9021/24	N*	0.02	
Human Serum	GHENT-SERUM	C	0.0077	3.9

Ni:Nickel

Urine	KL-142-I	C*	10	50
Human Hair	GBW-09101	C	3.47	11.5
Animal Blood	IAEA-A-13	N	1	
Human Hair	GBW-06701	C	0.83	23
Pig Kidney	BCR-CRM-186	N	42	
Single Cell Protein	BCR-CRM-274	N	0.38	
Urine (Spiked)	NIST-SRM-2670	N*	0.3	
Bovine Muscle	BCR-CRM-184	N	0.27	
Urine	KL-142-II	C*	0.2	37
Urine (Normal)	NIST-SRM-2670	N*	0.07	
Serum	KL-148-II	C*	0.05	40
Urine	STE-9021/24	N*	0.04	
Serum	KL-148-I	C*	0.03	66
Serum	STE-10017	N*	0.0032	

Pb: Lead

Human Hair	BCR-CRM-397	C	33	3
Human Hair	GBW-076o1	C	8.8	11.4
Human Hair	GBW-07101	C	7.2	9.7
Animal Bone	IAEA-H-5	C	3.1	18
Urine	KL-110-H	C*	1.1	18
Blood	KL-100-H	C*	0.95	21
Whole Blood	STE-056	N*	0.788	
Bovine Blood	BCR-CRM-196	C*	0.772	1.4
Bovine Liver	CZIM-LIVER	C	0.71	11
Urine	KL-110-M	C*	0.5	20
Blood	KL-100-M	C*	0.45	22
Whole Blood	STE-053	N*	0.306	
Blood	KL-100-L	C*	0.2	30
Animal Blood	IAEA-A-13	N	0.18	44

TABLE 3 (continued)

Material	Code	T	Concentration mg/kg or mg/L	Error %
Urine	KL-110-L	C*	0.15	47
Bovine Liver	NIST-SRM-1577b	C	0.135	11
Bovine Blood	BCR-CRM-194	C*	0.126	3.2
Freeze-dried Urine	GBW-09103	C	0.112	8
Urine (Spiked)	NIST-SRM-2670	C*	0.109	3.7
Animal Muscle (pork)	ARL/CL-AM	C	0.89	14.8
Urine	STE-9021/24	N*	0.088	
Whole Blood	STE-052	N*	0.062	
Single Cell Protein	BCR-CRM-274	C	0.044	22
Urine (Normal)	NIST-SRM-2670	N*	0.01	
Serum	STE-10017	N*	0.0027	

Se: Selenium

Material	Code	T	Concentration mg/kg or mg/L	Error %
Urine	KL-142-I	C*	20	37
Pig Kidney	BCR-CRM-186	C	10.3	4.9
Human Hair	BCR-CRM-397	C	2.0	3.5
Human Serum	GHENT-SERUM	C	1.05	4.8
Single Cell Protein	BCR-CRM-374	C	1.03	4.9
Human Hair	GBW-07601	C	0.60	6.7
Human Hair	GBW-09101	C	0.58	8.6
Urine	KL-142-II	C*	0.5	20
Urine (Spiked)	NIST-SRM-2670	C*	0.46	6.5
Freeze dried Urine	GBW-09103	C	0.44	13.6
Animal Muscle (pork)	ARC/CL-AM	C	0.394	7.9
Bovine Liver	CZIM-LIVER	C	0.325	4.3
Serum	KL-148-II	C*	0.25	24
Animal Blood	IAEA-A-13	C	0.242	12
Bovine Muscle	BCR-CRM-184	C	0.181	9.4
Serum	KL-148-I	C*	0.15	40
Skim Milk Powder	BCR-CRM-150	N	0.127	
Skim Milk Powder	BCR-CRM-151	N	0.125	
Milk Powder	NIST-SRM-1549	C	0.11	9
Human Serum	STE-10017	N*	0.09	
Human Urine	STE-9024	N*	0.09	
Skim Milk Powder	BCR-CRM-063	N	0.088	
Milk Powder	ARC/CL-MP	C	0.082	9.8
Human Urine	STE-9021	N*	0.049	
Bovine Serum	GBW-09131	C*	0.0389	5.9
Milk Powder	IAEA-A-11	C	0.0339	21
Urine (Normal)	NIST-SRM-2670	C*	0.03	27

TABLE 3 (continued)

Material	Code	T	Concentration mg/kg or mg/L	Error %
Tl: Thallium				
Urine	STE-9021/24	N*	0.01	
Bovine Liver	NIST-SRM-1577b	N	0.003	
Skim Milk Powder	BCR-CRM-150	N	0.001	
V: Vanadium				
Lobster Hepatopancreas	NRCC-TORT-1	C	1.4	21
Bovine Liver	NIST-SRM-1577b	N	0.003	
Skim Milk Powder	BCR-CRM-150	N	0.001	
Zn: Zinc				
Human Hair	BCR-CRM-397	C	199	2.5
Human Hair	GBW-07601	C	190	4.7
Human Hair	GBW-09101	C	189	4.2
Bovine Muscle	BCR-CRM-184	C	166	1.8
Bovine Liver	CZIM-LIVER	C	162	3.2
Pig Kidney	BCR-CRM-186	C	128	2.3
Bovine Liver	NIST-SRM-1577b	C	123	6.5
Animal Muscle (pork)	ARC/CI-AM	C	104	2.9
Animal Bone	IAEA-H-5	C	89	5.9
Single Cell Protein	BCR-CRM-274	C	42.7	2.3
Animal Blood	IAEA-A-13	C	13	7.7
Human serum	GHENT-SERUM	C	9.6	4.2
Freeze-dried Urine	GBW-09103	C	2.22	4.5
Serum	KL-146-I	C*	1.3	19
Serum	KL-146-II	C*	1.2	20
Human Serum	STE-10017	N*	0.9	
Human Urine	STE-9024	N*	0.8	
Human Urine	STE-9021	N*	0.64	

CONCLUSIONS

It is partly reassuring that the quality assurance of trace element analysis is now attracting increased attention, which is also demonstrated by a series of international symposia on biological and environmental reference materials (Wolf, 1985; Wolf and Stoeppler, 1987, 1988, 1990; Wagstaffe et al., 1993). Therefore, reference materials, such as those considered in this chapter, are being used by more and more by analysts. This is

a trend which certainly needs to be maintained. As far as the producers are concerned, the demand for new materials may be expected to continue indefinitely. In comparison with geochemical materials, of which approximately 170 were already available in 1984 (Govindaraju, 1984) the producers of biological reference materials have still a great deal of catching up to do.

Not only are new materials required, covering a wider variety of different kinds of matrix, but also, as indicated above, more data are needed on some elements of interest, such as aluminium and vanadium in existing materials. A further need is for producers of biological reference materials to pay attention in future to using a more uniform style of their data (i.e. how the certified values and their uncertainties, and information values, are defined and quoted); and also to reducing the magnitude of the uncertainties to levels that would be more useful in practice.

Last but not least, there will surely develop a great need in future for reference materials that have been certified with respect to specific chemical species. This will be a great challenge for both producers and users of biological reference materials. In order to meet this challenge in addition to the few already existing materials projects have been implemented to arrive at a variety of reference materials for element speciation purposes (e.g. Quevauviller et al., 1993).

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Statistics and data evaluation

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INTRODUCTION

The very first analytical chemists knew much about chemistry and little about statistics. In that time, say in the 17th century, the analytical procedures were rather complicated, thus the speed of analysis was not high and consequently very few test substances could be analyzed in a given time.

Later on, as the speed of analysis was enhanced, more samples could be analyzed and the laws of statistics could be applied to the enhanced number of samples. Moreover, as manufacturing of industrial products required quality control of the product, say the minimal mass of a piece of soap, statistical calculations were needed to ensure this quality.

The first question when dealing with a determination is: what do we want to analyze? Do we want a representative sample from an organ or do we want to find concentration differences within this organ? This and related questions will be dealt in the first part of this chapter. The determination itself often requires a calibration curve; statistics related to the bias, precision and detection limits will be dealt with in the second part. The third part is deals with data evaluation and concludes this chapter.

Before determination

Before the determination takes place the responsibilities of the analyst should be clear. In fact, in most cases the analyst should be responsible for the sampling procedure to make sure that samples which are to be analyzed do not differ from the material where they come from, i.e. the test substance must be representative of that material.

In case of trace elements no contamination must be introduced in the sampling procedure; this is dealt with in the chapters 'Sampling and Sample Storage' (A. Aitio and J. Järvisalo) and 'Sample Treatment' (B. Sansoni and V.K. Panday).

The analyst should also participate in the discussion about the sample mass and what should be calculated from the material to be determined, i.e. a mean value for the whole

material or different values for different sites or parts as in tissue studies. Further on, an estimation can be made about the sample size needed to detect differences between populations.

Sampling procedures

If we have a certain amount of material to determine, we can call the material in statistical terms a 'population'. In chemical analysis there is an infinite collection of samples which could be drawn from it. This population will always be infinite. It is important not to confuse the finite number of samples which is drawn with the infinite number of samples which could be drawn.

Three major random sampling techniques exist: 'systematic sampling', 'simple random sampling', and 'stratified random sampling'. (IPCS, 1992). All three techniques have a common purpose, namely that the samples are representative of the population or compartment to be sampled.

In simple random sampling the selection process is totally, unconditionally random. The disadvantage of this method lies in the possibility that the samples may unintentionally be clumped together, while other parts are not sampled at all. This may be particularly unfavourable when samples show a large variation.

Distribution problems may be solved by dividing the population or compartment into either equal segments where the investigator selects the interval systematically and in which segment a sample is to be taken, or into segments that are unequal in size or number (strata), and where at least one sample is taken in each segment.

Commonly simple random sampling and stratified random sampling are used and will be considered more in detail.

Simple random sampling

Example. A human population was studied in an area exposed to lead by a secondary smelter. This population was examined for the lead in blood concentration and compared with a control area population. First the blood samples of the exposed population were determined and subsequently the blood samples of the control population. Strange enough, the exposed area population levels were somewhat lower than the control area population levels. Further statistical analysis showed a significant negative correlation between the slope of the calibration curve of the method used and the mean values of the lead in blood concentrations. This showed that the calibration curve was 'overcorrecting' and that there was an influence of the calibration curve on the levels. A solution for this problem is random analyzing, i.e. the samples of the exposed and control areas should be determined at random every run.

It must be stresse that all conclusions we make about our determination are based on the assumption that we have obtained a random sample from our material. If we repeat the process of choosing an object at random from a population n times, the values $x_1, ...x_n$ of a random variable x so obtained will be a random sample.

Example. Nowadays it is very simple to obtain random numbers with pocket calculators. These numbers can be used for the random sampling, e.g. if we want to take 10 random samples from a population of, let us say 1000, we may get the following random numbers:

123, 930, 624, 806, 423, 455, 778, 289, 271, 133. If we have previously divided our population in 1000 units (people, area's, pieces) we have given all people etc a number from 1 to 1000, we know then that we may sample this population as stated before.

Random sampling is well suited to a problem in which we have no information about the natural grouping of the units of the population.

Stratified random sampling

Stratified sampling is appropriate to the case in which a certain logical or natural grouping may be expected within the population. Examples are the element or compound distribution in soil due to the water circulation, or the element distribution in biological tissues. Such a layer in the soil may be called a stratum. Here we want to know the mean of the stratum; the samples within the stratum are selected by a random method. Further on, the number of samples selected from any stratum should be proportional to the product of the total number of particles in the stratum N_i and the standard deviation σ_i.

Sample reduction

In practice we have to limit the quantity of material, as most modern methods require only minimal amounts of samples. Usually we reduce the particle size by mixing. Frequently, as a measure for the introduction of the additional variation by the subsampling procedure the size-weight ratio is introduced. This is defined as

$$b = \frac{weight\ of\ largest\ particle\ of\ impurity}{weight\ of\ reduced\ sample} \times 100$$

The scatter component due the subsampling is unlikely to exceed $b*x$, where x represents the percentage of impurity in the large sample.

Sample size and testing

Example. Suppose we want to take samples from two populations in order to estimate the difference. If we want, with two sided $(1 - \alpha)$ confidence, an error of at most in the estimated difference, then the sample size n should be

$$n > 2 \left(\frac{\sigma u_{1-\alpha/2}}{\delta} \right)^2$$

For $\alpha = 0.05$, $\delta = 2$ mg/L, $\sigma = 5$ mg/L, thus

$$n > 2 \left(\frac{5 \times 1.96}{2} \right)^2 \quad so\ n \geq 50$$

If it is possible to lower the standard deviation from 5 mg/L until 2.5 mg/L, only **12** samples would be needed. This shows the importance of a low standard deviation in relation to the sample size.

Blind determination

Example. When an analogue instrument is read, a trend will be visible that the digit 0 will be read more frequently than others as final digit, and, if a mark on 5 exists, the 5 will also be read more frequently than the others.

This is an example of how pre-information can bias the data evaluation. Another example is the well-known duplicate reading. If the duplicate value differs more than generally is accepted, a tendency is seen to repeat the measurement and to discard the 'outlier' without any motivation. We can state that besides the matrix composition the less information that analysts have, the more objective the result will be. It follows then that for each single determination the analyst should preferably receive only the matrix composition, e.g. "blood", "urine" etc., and the value of the randomly chosen test specimen number.

Reference samples and control samples

This material is dealt with in the chapter 'Interlaboratory and Intralaboratory Surveys and Reference Methods' by R.A. Braithwaite. The only statement we will make here is that a sufficient number of this kind of control specimen should be incorporated, e.g. one control specimen for 10 test specimens.

Determination

In the determination statistical methods may be used to improve the analytical method. Some important items will be pointed out, although the material will not be treated comprehensively, that relate to common day routine analysis.

Real replicates

From information theory (Pierce, 1961) it is known that a duplicate determination contains 100% more information than a single determination, a triplicate 50% more than a duplicate, and a quadruplicate 30% more than a triplicate. So the gain in information from single to duplicate determination is as large as the single determination itself! Here it follows that always at least a duplicate determination should be carried out. However, from a statistical viewpoint a duplicate determination is unsatisfactory as it contain too little test power.

Example. From the ISO statistical methods (1981) we learn that the one sided confidence interval for the population mean is defined as follows

$$\mu < \left(\bar{x} + \frac{t_{0.95}}{\sqrt{n}} \cdot s \right)$$

for the 95% confidence interval, where is μ population mean, $\bar{x}$ the sample mean, $t_{0.95}$ the Student-t test value at 95% confidence, n is the number of determinations and s the standard deviation.

Where $x = 12.5$ and $s = 0.8$ we get for a duplicate determination $n = 2$, $t_{0.95} = 6.314$ and $\sqrt{n} = 1.41$ thus $\mu < (12.5 + 4.46 \times 0.8)$, and $\mu \approx 16.1$ (a).

In case of a triplicate determination we have $n = 3$, $t_{0.95} = 2.920$ and $\sqrt{n} = 1.73$. Thus $\mu < (12.5 + 1.69 \times 0.8)$, and $\mu \approx 13.9$ (b). It will be clear that the confidence interval width for (b), the triplicate determination, is considerably smaller than for (a), the duplicate determination.

The triplicate determination is advocated in all circumstances where statistical methods for determinations are applied.

Example. It is common praxis in trace metal determination of organic substances that a destruction procedure is followed by the measurement of the trace element concentration in the digested fluid by atomic absorption spectrometry, voltammetry or another method. Often, a single digestion is carried out followed by a duplicate or triplicate measurement. The standard deviations of the method are given in this way by the scatter in the **measurement**. This, however, is underlined incorrect. For the standard deviation of the method we need a duplicate or triplicate digestion followed by a single measurement of each digested fluid. If later on we want to do analysis of variance to reveal differences in precision between the digestion and the measurement procedure, we need duplicate or triplicate observations of both the digestion procedure and the measurement (Bennett and Franklin, 1954).

Calibration curve

With many methods a calibration curve is needed. For statistical calculations a linear curve is preferable because of the simple statistics. Also the linear part of a further non-linear curve can be used for the calculations. Almost exclusively the method of linear regression by least squares approximation is used.

It is preferable to take at least four standards for the calibration curve. From both the standards and samples the homogeneity of the variances may be calculated. If the variances are homogeneous, the triplicate observations are normally distributed. The standard deviation s is the square root of the variance.

Example. For 4 standards A, B, C and D the following triplicate values are gained:

	Measurement	Variance s^2
A:	40, 41, 40	0.33
B:	85, 75, 80	25.00
C:	119, 120, 121	1.00
D:	151, 172, 162	110.33

It might be expected that the precision is not good, as variances range from 0.33-110.33 and a closer look to the procedure will be needed.

There are two methods commonly used for testing the homogeneity: the Cochran and the Bartlett tests. The first is an outlier test for variances and the second tests more for the scattering of variances. In the laboratory of the authors the Bartlett criterium (Pearson and Hartley, 1962) is preferred.

For testing linearity, analysis of variance is commonly used. An F-value can then be calculated from the ratio of the contribution of non-linear terms in the regression equation to that of the residuals.

Further on the 95% confidence interval width of the calibration curve may be calculated. If it is calculated according to Mandel (1964) it gives not only an impression of the imprecision of the standards, but also of the samples. Herber et al. (1983) described the least squares calculation, homogeneity of variances, linearity and confidence interval as applied in atomic absorption spectrometry.

Precision

The random error may be expressed as standard deviation (s, SD), relative standard deviation (RSD, formerly coefficient of variation CV or VC) or confidence interval width. In our opinion using the confidence interval is the best procedure, especially when the earlier mentioned Mandel (1964) method is used, where the error in standards as well as samples are incorporated. For comparison purposes the 95% confidence interval width of an observation will be about $4*s$. If one wishes to use separate standard errors for samples and standards, e.g. if the calibration curve has a much smaller error than the standards as sometimes in the case of aqueous standards, the total error can be calculated as follows:

$$s_T = \sqrt{s_{st}^2 + s_{sa}^2}$$

where s_T is the total error, s_{st} is the standard error of the standards and s_{sa} of the samples.

Bias

Systematic errors cause a bias from the 'true' values which are hardly expressible in statistical terms. Most statistical handbooks neglect this analytical item, although it is a question of utmost importance for the analyst. The first question is always whether the error is systematic or random. This question cannot be answered in one determination, thus it will be dealt with later.

Bias was formerly described in terms of 'accuracy', 'inaccuracy' or 'deviation from the true result'.

Detection limit and blank

Usually the detection limit may be calculated in a simple way as follows:

$$Y = \sqrt{\frac{\sum y_i^2}{n}}$$

where y_i is the noise around the baseline measured peak-peak during the appearance time of the peak, n the number of equidistant measurements, preferably ≥ 6, and Y is the root mean square of y_i. Then a detection limit (DL) with an arbitrary error might be chosen, e.g. with an error of 50%, DL $= 2Y$, and with an error of 33%, DL $= 3Y$.

In case of blanks which contribute substantially to the measurement, the above mentioned procedure is not adequate. In this case the detection limit may be calculated as follows next.

First the variation of the blank is calculated by

$$B = \sqrt{\frac{\sum b_i^2}{n}}$$

where B is the mean blank value, b_i the blank value and n the number of measurements, preferably ≥ 6.

Subsequently the total fluctuation is calculated by error propagation of the noise and the blank:

$$TF = \sqrt{Y^2 + B^2}$$

where TF is the total fluctuation. With this formula the detection limit can be calculated again.

With an error of 50%, DL $= 2TF$. If $Y < B$ (e.g. $Y < 0.1B$), $TF \approx B$ and the detection limit will be dependent on the blank, only. If the blank varyies, the standard deviation of B will also vary and thus the detection limit. This is an undesirable situation: the variation of blank and detection limit must always be kept between narrow limits.

Example. In the determination of mercury by a cold vapour atomic absorption spectrometry method we find blanks, caused by impurities in the used chemicals. The blanks are expressed in μg/L: 1.0, 0.6, 0.7, 1.2, 1.1, 0.8, 0.9, 0.6, 0.7, 1.1. It follows that

$$B = \sqrt{\frac{1.0^2 + 0.6^2 + 0.7^2 + 1.2^2 + 1.1^2 + 0.8^2 + 0.9^2 + 0.6^2 + 0.7^2 + 1.1^2}{10}} = 0.89$$

If Y (root mean square noise) $= 0.2$,

$$TF = \sqrt{0.2^2 + 0.89^2} = 0.91$$

The difference between these two figures is $0.91 - 0.89 = 0.02$ μg/L. Thus the noise plays a minor role only. If DL $= 2TF$, the detection limit will be 1.82 μg/L. Without blank, however, the detection limit would be DL $= 2Y = 2 \times 0.2 = 0.4$ μg /L, or an improvement of a factor of 4.5!

Post determination

The first data evaluation should regard the quality of data. Well-known methods are quality control charts (WHO, 1981). Another method is using outlier statistics. The first

method is preferable because trends also can be made visible, moreover if several control samples are used the reliability of the control scheme is higher than that of outlier statistics. The Shewhart method (Reed and Henry, 1974) is the most widely used, but a cumulative sum chart ('cusum chart') is used sometimes as well (Reed and Henry, 1974).

Another method which fills the gap between analytical chemistry and the usage of the data for further calculating is the presentation of the data in a frequency-concentration diagram, e.g. a histogram. This method may indicate some peculiarities in groups of data.

Control chart

The Shewhart control chart can be made with own samples from a pool, or with control samples commercially available, or preferably, with certified reference samples. Such a control chart can be made in three different ways, i.e. (1) the allowable range of the manufacturer is used (2) the 2 or 3 s range or 95% confidence interval width of the mean of the determinations is used and (3) a fixed percentage is used as allowable range (Fig. 1). In the last two cases the graphic interpretation is rather simple: partial or complete overlap between an allowable range and s, respectively confidence interval width, will be considered as 'no difference'. No overlap means a difference between control sample and allowable range. The s, respectively confidence interval width of the determination of the control sample, should always be given. Control charts are mostly used for bias assessment, but they may also be used for precision. In the last case, we may construct a range chart. The range $\bar{R}$ is calculated by

$$\bar{R} = \frac{R}{n}$$

where R is the range of the replicate determination and n the number of samples. With the aid of tabulated factors R we may calculate upper and lower limits for the precision (Bennett and Franklin, 1954).

Example. We obtained the following results from quadruplicate determinations from 30 samples: $\bar{R}$ = 314.5; upper limit = tabulated factor D_4 x $\bar{R}$ = 2.282 x 314.5 = 717.7; lower limit = tabulated factor D_3 x $\bar{R}$ = 0 x 314.5 = 0. With the aid of simple linear regression a trend can be tested.

Outliers

Outliers can be tested by three different methods, i.e. Bartlett's homogeneity of variance test, Cochran's one sided outlier test and Hartley's variance ratio test. The ISO (1986) recommends Cochran's test, because (1) the two other tests cannot be applied when one of the variances in a set is zero and (2) the two other tests are very sensitive to the value of the smallest variance. Although in our opinion a zero variance may be easily overcome by setting the variance to a small finite value, the other argument is certainly true for outliers. Cochran's criteria C is given by
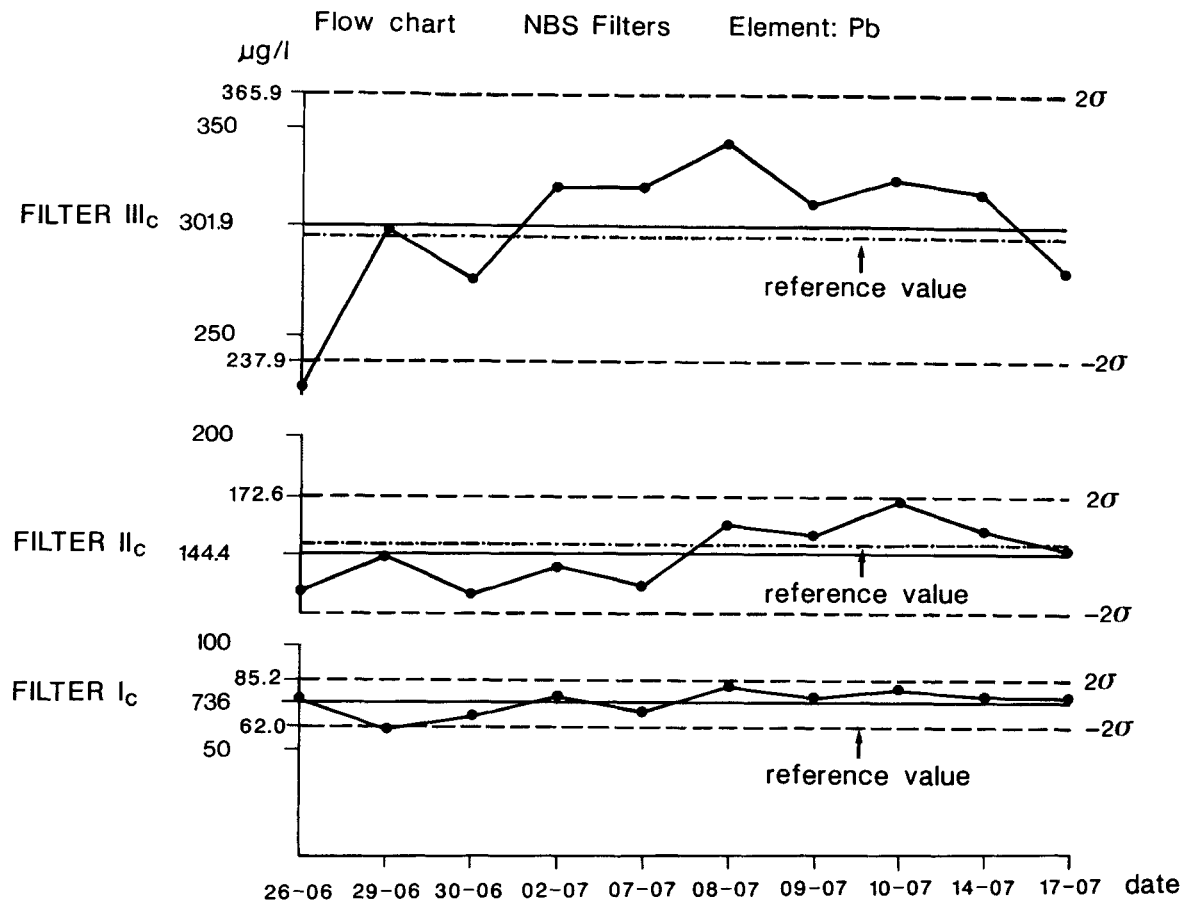
Figure 1a. Shewhart (control chart) of control samples for the determination of lead in filter material. Concentration in μg/L, filters from NBS (currently NIST). Limits set at 2 s of reference value.
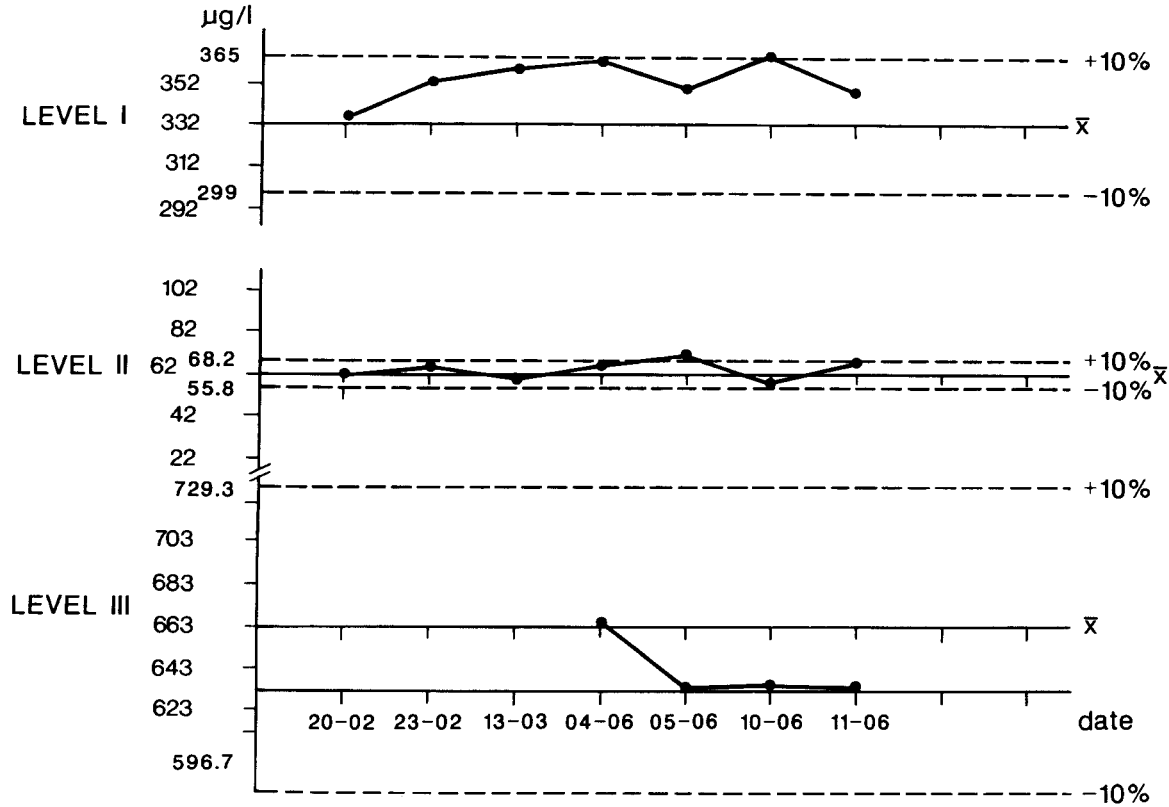
Figure 1b. Shewhart (control chart) of control samples for the determination of lead in blood. Concentration in μg/L, blood samples made in own laboratory. Limits set on 10 % of mean value of control samples determined so far.

$$C = \frac{s_{max}^2}{\sum_{i=1}^{p} s_i^2}$$

Value s_{max} is the highest value in the set, p is the set of standard deviations S_i of n replicate test results.

It must be remembered that an outlier test should always be followed by an examination of what caused this outlier. In our opinion the use of outlier tests is limited; a better way to incorporate all results is to employ rigid or robust statistics, i.e. the use of (log) transformations (geometric means) or medians instead of arithmetic means.

Analysis of variance

Analysis of variance, especially the simple one way form is very useful to reveal differences in concentration levels in the determination where they should not be. Examples are the problem of contamination in trace element analysis (if the contamination is caused by the chemicals used, the destruction procedure, or the vessels). Other differences which can be tested are differences between determination or between procedures, etc.

Differences in variance are tested by using an F-distribution, representing the quotient of the mean square between samples and the mean square within samples. This is called the sample variance ratio. This material is comprehensively treated in the statistical handbooks (Bennett and Franklin, 1954).

Distributions

All statistical analyses treated so far only can be used assuming the data show normal distribution. This type of distribution appears in analytical chemistry most often as limiting distribution. If a distribution of data is not normal, a simple logarithmic transformation of the data sometimes is sufficient to get a normal distribution. The mean of the transformed data distribution may be transformed back and is called then the geometric mean in contrary to the commonly used arithmetic mean.

In statistical analysis involving normal distributions some other types of distributions are encountered frequently. The t-distribution is encountered e.g. in the calculation of confidence intervals in various situations. Its limiting distribution is the standard-normal distribution. The χ^2-distribution is the sum of squares of several standard-normal distributed variables. It may be encountered in tests on normality of data.

The F-distribution is in fact the distribution of the ratio of two variances. It is encountered e.g. in regression analysis.

The Poisson distribution arises when counting relatively seldom events such as lightning striking or radioactive processes as in the case of radiochemistry.

This discrete distribution is given by
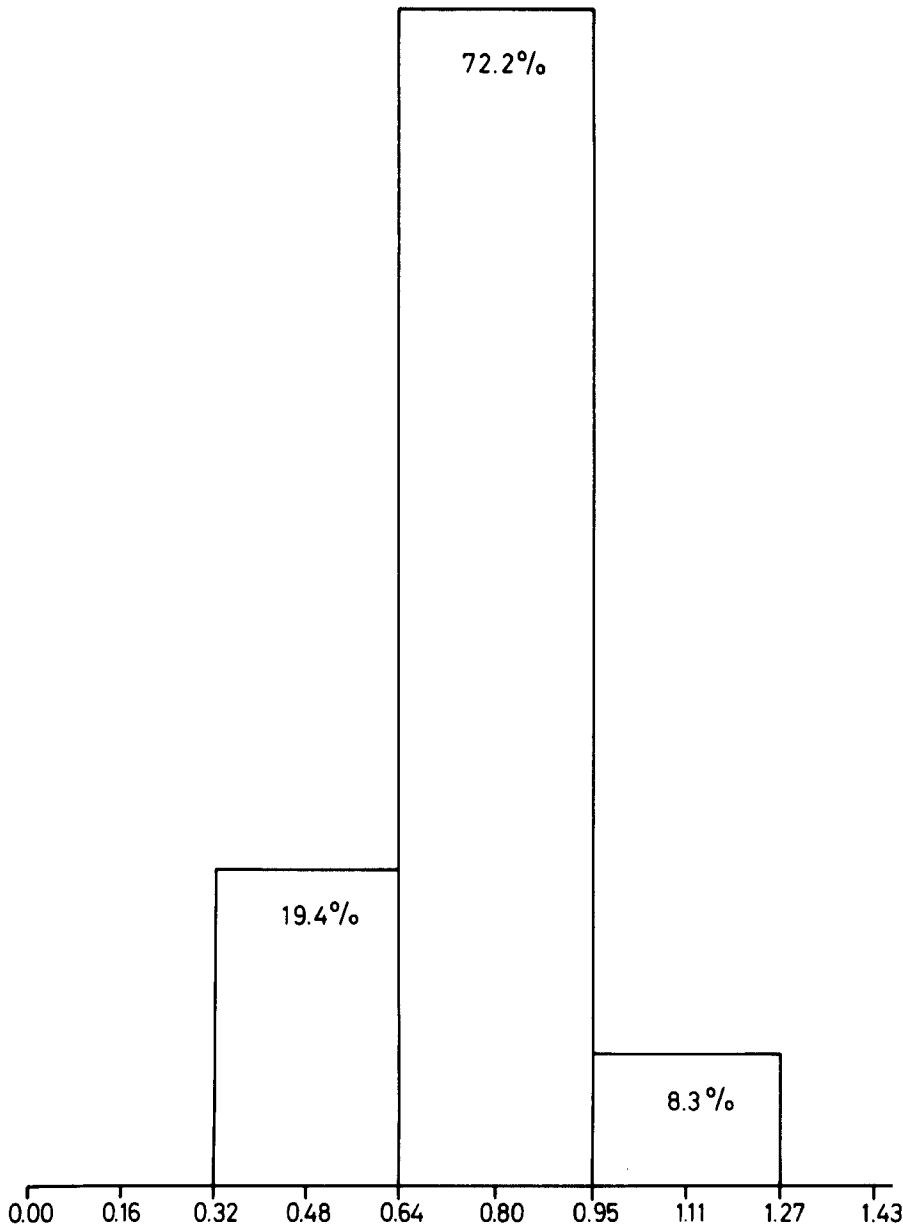
Figure 2a. Histogram of the distribution of the concentration of zinc in serum in 27 samples. Concentration in mg/L on x-axis. Conventional histogram (after Herber and Pieters, 1982).
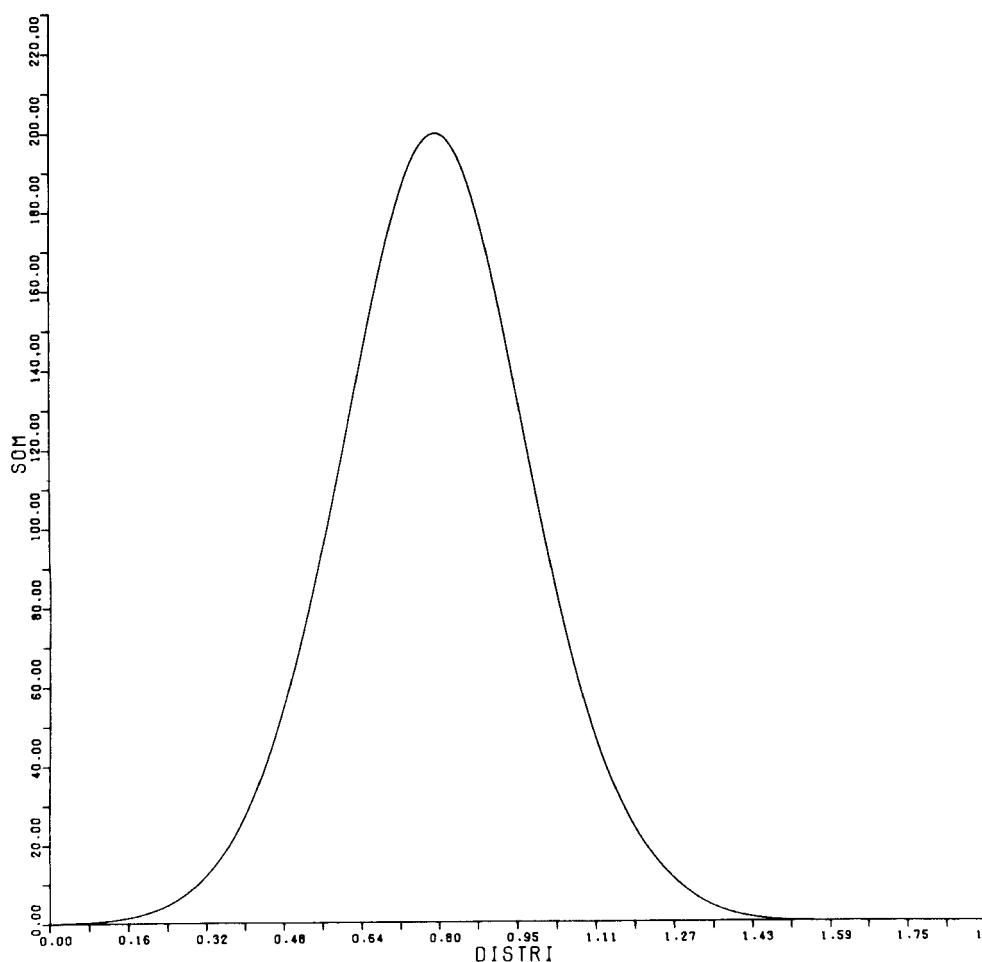
Figure 2b. Distribution of the concentration of zinc in serum, using the standard deviations of each individual determination and summation. See further Fig. 2a. Distribution shows perfect Gaussian character.

$$p(x) = \frac{e^{-\lambda}\lambda^x}{x!} \quad \text{for } x = 0, 1, 2 \ldots$$

where x is the number of events which occurred in a given observation period and λ the expected number of events in such a period. A useful property of this distribution is that the variance is equal to the mean which is the expected number of events. Thus - $s_{Poisson} = \sqrt{\lambda}$.

This distribution can be expected in case of solid sampling analysis when rare particles of a high analyte content are encountered (Kurfürst, 1991).
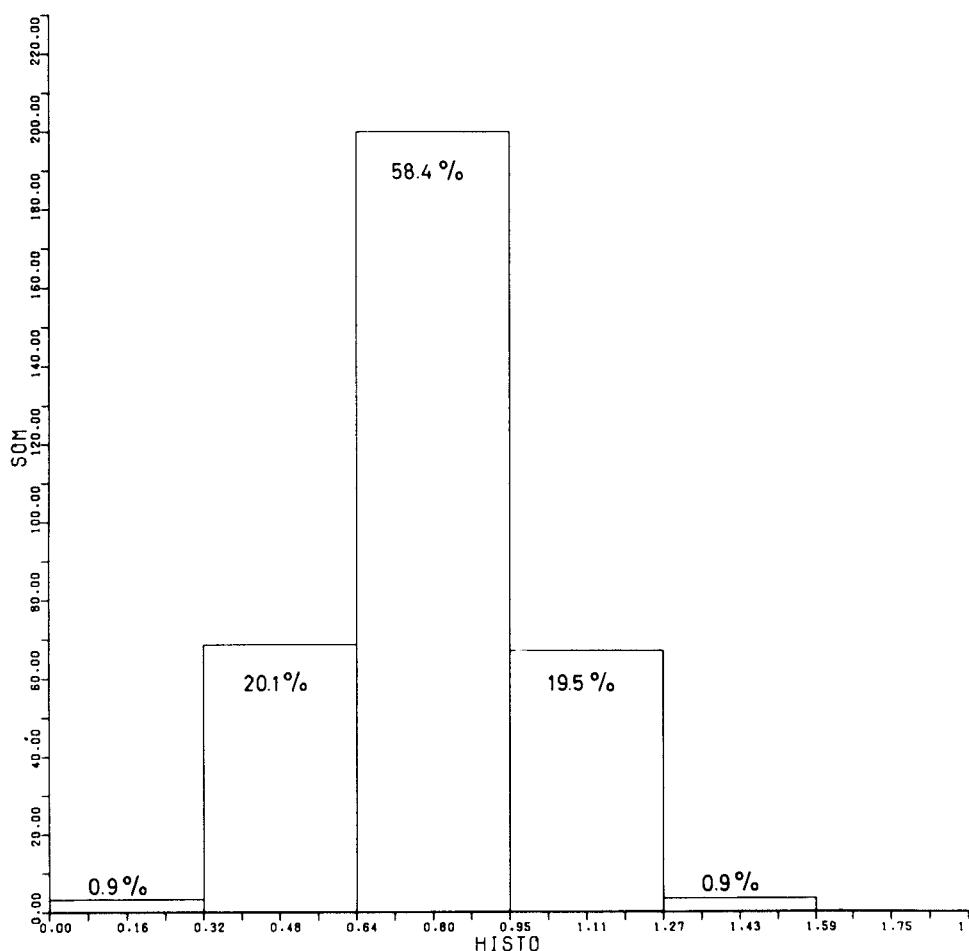
Figure 2c. Histogram of the distribution on zinc in serum, using the standard deviation of each individual determination. See further Fig. 2a. Histogram shows nearly symmetrical character, in line with Fig. 2b.

Histograms

Other useful types of distributions are the 'simple adding distributions'. Examples are the cumulative frequency distribution and the histograms. Herber and Pieters (1982) made an adding distribution and histogram using not only the data of the analysis, but also the precision of the determination. This may lead to a better insight into the real character of the distribution. Fig. 2 gives an example.

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Chapter 13

Aluminium

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ABSTRACT

Aluminium monitoring is of vital importance to patients with chronic renal failure being treated with intermittent hemodialysis. Aluminium sources in these patients are dialysate contamination and the ingestion of aluminium-containing medications. The development of unbiased and precise methods. for the determination of aluminium in biological materials is crucial to monitoring these hemodialysis patients after areas of aluminium toxicity.

Several techniques have been used for the determination of aluminium, the best being neutron activation analysis, inductively coupled plasma emission spectrometry and graphite furnace atomic absorption spectrometry. The latter technique has been most widely used. Separation of aluminium species in plasma can be accomplished by ultrafiltration and by gel permeation chromatography. Several forms of aluminium appear to exist in plasma.

Many types of specimens have been used to assess biological effects of aluminium; serum and bone samples have provided the best clinical information. Collection and processing of specimens must be carried out in such a manner as to minimize contamination.

INTRODUCTION

Iatrogenic aluminium poisoning is now one of the most important clinical problems involving trace metal toxicity. Several thousand determinations are performed daily around the world in order to monitor exposure to aluminium in patients with chronic renal failure being treated with intermittent hemodialysis.

Aluminium is the third most abundant element in the earth's crust and industrial use of this metal is in the tens of millions of tons per year. Aluminium has found applications in construction, electrical equipment, furnishings, transportation, containers, pharmaceuticals and many other items encountered in daily life. Exposure to aluminium,

and impairment of the normal efficient renal mechanism of excretion of aluminium. They subsequently reported the effects of aluminium poisoning in uremic and non-uremic rats, after modest doses of oral and parenteral aluminium salts (Berlyne et al., 1972). In these animals aluminium poisoning was associated with periorbital bleeding, lethargy, anorexia, and death. These features were associated with significant increases in the aluminium content of serum, liver, heart, striated muscle, brain, and bone tissues. Berlyne and his colleagues (1972) proposed that, because of its massive deposition in these various tissues, aluminium might account for some features of the clinical syndrome of uremia.

Aluminium salts are extensively used in the therapeutic management of the hyperphosphatemia which arises in chronic renal failure. Aluminium is absorbed from the gastrointestinal tract; normal subjects have a rise in serum concentration followed by excretion in the urine (Mauras et al., 1982). There is now substantial evidence that, in patients with end-stage chronic renal failure managed by long-term intermittent hemodialysis, there is hyperaluminemia with accumulation of aluminium in various tissues. The excess in serum and tissue results from intestinal absorption of aluminium salts taken by mouth and from passage of aluminium across the dialysis membrane. The aluminium content of the dialysate depends on the content of the water with which it is made. Aluminium is normally present in raw water; the concentrations are usually low in ground waters and are almost always high in surface waters (Miller et al., 1984).

Some domestic tap-water contains aluminium in high concentration, either naturally or because aluminium has been added as a flocculant in the purification process. Acid rain markedly increases the "natural" aluminium content of water. In chronic renal failure Mayor et al. (1981) have proposed that parathyroid hormone may contribute to the hyperaluminemia by increasing intestinal absorption and by influencing tissue distribution. The interrelations between parathyroid hormone and the homeostasis of aluminium and calcium remain to be defined. In aluminium poisoning the secretion rate of parathyroid hormone may be either reduced (Canata et al., 1983) or unchanged (Biswas et al., 1982).

Clinical and other biochemical studies of aluminium almost always are dependent on the availability of unbiased and precise analytical methods. All biological specimens are complex mixtures of a multitude or organic and inorganic constituents making the detection of a trace constituent, such as aluminium, extremely challenging. The analyst is faced with the problems of detecting aluminium in the μg/kg range, and must be able to accomplish collection, storage, processing, and final analysis without outside contamination from this ubiquitous element. The abundance of aluminium in the environment complicates efforts to provide a contamination-free system.

Many problems have existed with analytical methods of the determination of aluminium, and have been detailed by Cornelis and Schutyser in a review of analytical problems related to these assays (Cornelis and Schutyser, 1984). This review included data published since 1974 on aluminium in serum (or plasma) and urine of normal healthy individuals. In one group, 19 mean values of serum aluminium were listed for their respective normal range studies. These mean values ranged from 2.1-4.2 μg/L. Other reports have listed normal mean concentrations ranging from 72 to 1460 μg/L although obvious interferences were present in these methods. The literature survey of Cornelis and Schu-

tyser (1984) also provided normal whole blood mean aluminium concentrations which ranged from 1.6 to 22.1 μg/L, and urine levels which varied between 4.7 and 1700 μg/L. There were three studies for whole blood and seven reports for urine. In the absence of carefully controlled interlaboratory surveys with well characterized quality control materials, the use of each laboratory's normal range provides a useful indicator of interlaboratory variability. This variability reported by Cornelis and Schutyser (1984) is undoubtedly due to major analytical problems rather than to biological variation.

Considerable improvement in analytical methods for aluminium has been achieved recently. At least two excellent interlaboratory surveys have been introduced to aid the analyst by providing materials which are analyzed in several laboratories.[*] Also the recognition that normal human serum (or plasma) aluminium levels are less than 10 μg/L has helped many laboratories to identify contamination and analytical problems. This present review surveys several aspects of the analytical biochemistry of aluminium. Collection, contamination avoidance and a wide variety of analytical applications are included.

ANALYTICAL METHODS

Chemical and Physiochemical Methods

Aluminium can be determined using gravimetric, titrimetric, photometric and fluorimetric methods. Interferences from other metals and contamination of reagents with aluminium are major problems with these techniques. All of these methods have as a basic requirement that the aluminium is isolated from interfering metals. The procedures used to do this isolation include either separation of the metals from aluminium or masking them in a manner that inhibits their interference. All of these methods were originally designed to determine the aluminium content in water, or metal alloys. All determinations were performed in an aqueous environment. A spectrophotometric determination of aluminium in water utilizing catechol violet is still in general use by the regional water authorities in the United Kingdom. However, the complexity of the serum matrix, with its various constituent metals, potentially interferes with these methods. The proteins in serum would also precipitate with most of the reagents used in these methods, adding further to the difficulties of chemical or physiochemical analysis. For these reasons, together with their poor sensitivity, the various chemical and physiochemical methods are unsuitable for aluminium determinations in serum and other biological materials.

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X-Ray Fluorescence

X-ray fluorescence is associated with the K-shell electrons of metals. A thin film of the sample containing metals is placed on a small mylar sheet and dried. The sample is then bombarded with an electron beam and when an incident electron interacts with a K-shell electron in the metal, the K-shell electron is elevated to an unstable orbital state. As the K-shell electron returns to its stable orbital, it emits energy in the form of X-rays which are characteristic of the metal involved. These X-rays can be counted by an appropriate detector and the energies of the X-rays correspond to different metals. Even though this method is very specific and capable of measuring aluminium, it does not appear to be sensitive enough in its present state to detect the trace levels of aluminium in serum (Sorenson et al., 1974). However, one form of X-ray emission spectrography, that of electron probe X-ray microanalysis, has been used effectively to localize aluminium in both bone and brain tissues (Smith and McClure, 1982). Localization of aluminium in tissues will be discussed briefly in a later section.

Neutron Activation Analysis

Several investigators have used neutron activation analysis (NAA) to determine the aluminium content of biological specimens both with and without some chemical processing. Instrumental neutron activation analysis involves the bombardment of a sample with neutrons and the measurement of the radioactivity induced by nuclear reactions. No chemical processing is required. Upon activation ^{27}Al (100% isotopic abundance) forms the radioactive ^{28}Al nuclide by a (n,y) reaction. There are a number of attractive features in this technique which include excellent sensitivity with relative independence from matrix effects and interferences. Also, there is relative freedom from contamination since the sample is analyzed directly with minimal handling. One major problem is the need to correct for fast neutron reactions on Phosphorus which also produce ^{28}Al. A detailed description of the technique from the analysis of brain tissue has been reported recently (Ehman et al., 1983).

Other NAA methods involve the chemical separation of interfering ions (Blotcky et al., 1976); in these the short half-life (2.24 minutes) of ^{28}Al makes post-irradiation separations a problem. Pre-irradiation separation techniques have potential problems of contamination and losses during the separation phase.

The facilities required for NAA and the obvious problems associated with either chemical separations or the spectral interferences in the instrumental NAA methods make this technique impractical for most investigators. Also the NAA cannot be classified as a definitive or reference method since there are obvious interferences such as phosphorus which require some correction factor.

Atomic Emission Spectrometry

Aluminium can be determined by emission spectrometry using either a nitrous oxide acetylene flame or an argon plasma.

Flame Emission

A method has been described (Ihnat, 1976) that was satisfactory for high aluminium levels, but was not sufficiently sensitive to detect the low concentrations that exist in serum. The difficulty with the nitrous oxide acetylene flame method is that this type of flame is not hot enough to provide the energy needed to ionize all of the aluminium and, thus, is unsuitable for determination for trace levels.

Inductively Coupled Plasma

Recently the lack of energy in flame emission has been overcome by the use of inductively coupled plasma (ICP) as an excitation source. In this technique a stream of argon is ionized by a radio frequency and the plasma of the ionized argon which is formed can reach temperatures of 4000°C. Inductively coupled plasma emission spectrometry is a multi-element technique which is relatively free of chemical interferences. The matrix problems which exist in atomic absorption spectrometry are eliminated in ICP due to the very high excitation temperature of the sample. Thus, the technique should be particularly useful for the determination of refractory elements such as aluminium and silicon. Inductively coupled plasma methods have been reported for the measurement of aluminium in biological materials (Lichte et al., 1980; Schramel et al., 1980; Mauras and Allain, 1985) and could be an excellent alternative to graphite furnace atomic absorption spectrometry for those laboratories possessing the appropriate instrumentation. A major problem with using the argon-plasma technique is the intense and broad emission of calcium which increases the aluminium background and can raise the detection limits for this element. Correction for this problem is hampered because the effect of calcium varies with its concentration.

However, the sensitivity of ICP methods probably is comparable to graphite furnace atomic absorption and is reported to be about 2 μg/L (Lichte et al., 1980) although relatively poor sensitivity (10 μg/L) also has been reported (Schramel et al., 1980). A report (Mauras and Allain, 1985) describes an excellent study of the application of ICP to the analysis of blood, dialysis fluid and water. The methods described in this report (Mauras and Allain, 1985) are automated by use of an automatic sampler. There is computer-controlled displacement of the entrance slit allowing easy and precise measurement of the background for each sample. Variations in emission signal intensity in matrix of different compositions are cancelled by addition of cesium as a matrix modifier and by using gallium as an internal standard. The detection limit of the method is 0.3 μg/L in pure solution with a large range of linearity and excellent precision. This technique offers good potential as a reference method for aluminium determinations. One drawback to ICP, however, is the relatively high cost of the instrumentation which will limit its use in many routine laboratories.

Atomic Absorption Spectrometry

Atomic absorption spectrometry (AAS) has been widely used to determine biological materials for aluminium content. Flame techniques, even with the hotter nitrous oxide acetylene flame, do not perform as well as the graphite furnace methods. However, flame atomic absorption methods have been used to analyze brain and cerebrospinal fluid (Krishnan et al., 1972), rat tissues (Mayor et al., 1980), food, urine and feces (Clarkson et al., 1972), heart muscle (Chipperfield et al., 1977), and plasma and tissues (Berlyne et al., 1972; Berlyne et al., 1970; Weinberger et al., 1972).

The greatest degree of success of any technique for the determination of aluminium in biological specimens has been with graphite furnace atomic absorption spectrometry (GF-AAS). In this technique the sample is placed in a graphite tube mounted in the light path of the spectrometer. The source of the light is a hollow cathode lamp that contains the metal being analyzed and emits characteristic wavelengths. First the graphite tube is heated with direct current to dry the sample at a low temperature, then the sample is ashed to destroy organic matter and burn off inorganic species that may interfere, and finally the temperature is quickly raised and the metal under analysis vaporizes and absorbs the light being passed through the graphite tube. Advantages of the graphite furnace include: (a) sample pretreatment can usually be eliminated, (b) sample require-ments are small (2-100 μL), (c) graphite furnaces are capable of attaining the high temperature needed to form ground state atoms, and (d) the atoms stay in the light path for a relatively long time which results in increased sensitivity.

Several problems may be encountered in the GF-AAS determination of aluminium, and include difficulties with untreated graphite tubes (Alderman and Gitelman, 1980), matrix interferences (Alderman and Gitelman, 1980; Gorsky and Dietz, 1978), and stand-ardization procedures (Gorsky and Dietz, 1978).

An investigation of two GF-AAS methods has been carried out in the authors' labora-tory (Brown et al., 1984). The first was a conventional approach using direct analysis of serum using a stabilized temperature platform in the graphite tube. The study demon-strated the necessity of using the method of standard additions as a means of standardization, since there were profound differences in the slopes of standard curves constructed from aqueous standards, sera from normal individuals, and sera from uremic patients. Thus, considerable analytical errors would result from using aqueous standards or standards made up in normal sera as a means of standardizing assays for uremic patients. The standard additions method allows each serum sample matrix to serve as its own standard and, therefore, provide more accurate analyses.

Leung and Henderson (1982) have not been able to confirm these findings of grossly different curve slopes for different types of serum and standard samples. Bettinelli et al. (1985) made a thorough evaluation of the direct measurement of aluminium in serum using GF-AAS with the stabilized temperature furnace with the L'vov platform. Their rec-ommendation was to use pyrolytically coated graphite tubes and to keep the method as simple as possible with minimal sample pre-treatment. Slavin (Personal Communication) also has been unable to observe major slope change problems between aqueous and

serum matrix samples and recommends a procedure similar to that of Bettinelli et al. (1985), Leung and Henderson (1982), and Brown et al. (1984). Most GF-AAS procedures have been developed on Perkin-Elmer instruments with the use of auto-sampling to improve precision. Pyrolytically coated graphite tubes are recommended together with a pyrolytic graphite platform. Argon is preferred over nitrogen as the purge gas since argon produces a larger and less variable signal. Some type of background correction is recommended and the Zeeman correction system probably provides the most sensitive and reliable results. In the direct methods for serum analysis standards and serum samples are diluted with an equal volume of an aqueous solution containing $Mg(NO_3)_2$ (2 g/L). The autosampler is programmed to deliver a 15 μL aliquot of the sample onto the platform for final analysis. The GF-AAS program used in the authors' laboratory on the Perkin-Elmer Model 5000 instrument, is given in Table 1.

Leung and Henderson (1982) prepare their standards using a serum pool containing a minimal amount of endogenous aluminium, whereas Bettinelli et al. (1985) recommend aqueous standards. The use of simple aqueous standards is to be preferred provided that there are no matrix effects from serum.

The present authors have developed a procedure which minimizes any potential matrix effects by protein precipitation (Brown et al., 1984). This protein precipitation technique was originally developed for serum nickel (Sunderman et al., 1984) and markedly reduces matrix effects in the final atomic spectroscopic analysis. Briefly the procedure is as follows:

One mL of serum is pipetted into an acid-washed 4.5 mL polyethene centrifuge tube (cat. no. 477, Walter Sarstedt, Inc., Princeton, NJ 08540). The tube is positioned on a Vortex mixer and mixing is begun at medium speed. 50 μL of ultrapure concentrated nitric acid is pipetted into the middle of the vortex. The tube is capped and mixing continued for one minute followed by heating for 5 min in a 70°C water bath, mixing for 10 s by vortexing, and centrifuging for 10 min at 900 x g. The supernatant is pipetted into a teflon sampling cup with an acid rinsed pipet tip and analyzed using GF-AAS with a stabilized platform. This protein precipitation technique is precise (5% RSD) and is linear to 120 μg/L.

For urine analysis sample aliquots are diluted 1:1 with distilled water before application to the AAS stabilized temperature platform (Leung and Henderson, 1982). Fecal analysis require considerably more complicated preparation steps than serum or urine. The procedure developed in the author's laboratory (Brown et al., manuscript in preparation) is summarized as follows: Frozen specimens are thawed and distilled water is added (1 mL per 2 g feces) and the sample is homogenized in a sealed container on a paint shaker. A 10 mL aliquot is ashed at 550°C in a muffle furnace, dissolved in dilute HNO_3 and analyzed by GF-AAS.

Solid tissues must be homogenized, dried, ashed, and/or dissolved to produce a liquid sample prior to GF-AAS analysis. Bone samples for determination are washed free of marrow by a strong stream of distilled water. Any existing fat or muscle is scraped with an aluminium-free obsidian scapel or blade found to be aluminium-free. The bone sample can then be processed by several methods to obtain a solution for injection into the

TABLE 1

INSTRUMENT PARAMETERS FOR GF-AAS ALUMINIUM DETERMINATION OF SERUM

Step	Description	Temp, °C	Ramp, s	Hold, s
1	Dry	130	1	45
2	Oxygen Ash	600	30	55[1]
3	Purge	600	1	25
4	Char	1700	15	25
5	Atomize	2400	0	6[2]
6	Clean	2600	1	6
7	Cool	20	1	20

[1] Ar flow = 0 mL/min
[2] O_2 used as flow gas

graphite furnace. In one method, the bone sample is placed into a quartz Kjeldahl flask. 5 mL of ultrapure concentrated nitric acid is added and the flask is electrically heated to 200° C. Destruction is complete when a clear solution is observed. The remaining liquid is evaporated, the flask allowed to cool, and 1 mL of concentrated nitric acid added. The liquid is quantitatively transferred to a volumetric flask and diluted with distilled water and analyzed by GF-AAS (D'Haese et al., 1985). A second method for processing bone samples is extraction of aluminium in the sample with a saturated solution of disodium ethylenediaminetetraacetate (EDTA) (LeGendre and Alfrey, 1976). The bone is washed as described above, allowed to air dry, ground in a Wiley mill, passed through a sieve, partially defatted with petroleum ether, reground, and passed through a sieve again. Pre-weighed samples are placed into 5 mL of the EDTA solution and mixed for 2 to 4 hours. The supernate is analyzed for aluminium and concentrations are calculated against standards prepared in the EDTA solution. Bone samples can also be ashed in the same manner as for fecal samples, as described earlier. This method allows for the calculation of aluminium based on wet weight or dry weight.

Soft tissue samples, such as brain, liver, muscle, etc. must be homogenized before processing, and this can be accomplished easily by pummelling the tissue in a "Stomacher" blender (Fisher Scientific). Distilled water 5 mL is added to the bag with the tissue, the bag sealed, and placed in the blender and blended 5 to 15 min. which completely homogenizes the sample (Sunderman et al., 1985). The homogenate can then be processed as described for the fecal samples. Tissue samples can also be processed by the EDTA method described for bone. Brain samples are dried and ashed, then extracted with EDTA. Muscle samples are dried, ground, extracted in solvents, redried, and extracted in EDTA (LeGendre and Alfrey, 1976). Tissue samples can be processed (after homogenization) with hot nitric acid in a Kjeldahl flask as described above. Soft tissue also can be processed for aluminium analysis by using tetramethyl ammonium

hydroxide (TMAH) as a dissolving solution (Stevens, 1984). Tissue samples are dried to constant weight, 2 mL of aqueous TMAH solution is added, dried in a hot-air oven (90°C) for 1 to 2 hours, during which the solution is mixed occasionally. This procedure will completely dissolve the tissue. The solution is cooled, diluted with ethanol, and mixed well. Working aluminium standards are prepared by adding aliquots of an intermediate standard to 2.0 mL of aqueous TMAH and diluting to 10 mL with ethanol.

Separation of Aluminium Species in Plasma

Kaehny et al. (1977) studied aluminium transfer during hemodialysis from a dialysate with a low aluminium concentration to blood with an elevated concentration. Aluminium appeared to be strongly bound to a serum or plasma component, but the binding sites apparently were saturable, since plasma aluminium values reach a plateau during the dialysis procedure.

Using ultrafiltration techniques, several investigators have reported protein-bound and nonprotein-bound fractions of plasma aluminium. Lundin et al. (1978) studied the partition of aluminium in the plasma of 10 normal individuals using ultrafiltration with membranes having a cutoff of 6,000-8,000 daltons. The results obtained showed that the protein binding averaged 59% of total serum aluminium.

Elliott (1978) in a brief communication reported that more than 70% of the aluminium in blood was present in the plasma compartment. In normal subjects, they reported that a very small proportion of the plasma aluminium was ultrafiltrable. These findings were not consistent with thos of Lundin et al. (1978). In the study of (1978) the tendency was for the ultrafiltrable fraction to decrease as the total plasma aluminium concentration fell below 200 μg/L. At high plasma aluminium levels, studies using polyethene glycol and direct ultrafiltration indicated that 60-70% of the aluminium was bound to high-molecular weight proteins, 10-20% was bound to albumin, and 10-30% was ultrafiltrable.

Graf et al. (1982, 1981) performed in vivo ultrafiltration studies on patients during hemodialysis. Their results revealed an ultrafiltration fraction of about 20% of total plasma aluminium, suggesting that 80% of the aluminium was protein bound.

It is apparent that more detailed information is needed of aluminium distribution in the plasma of normals and patients on chronic intermittent hemodialysis. Such information would clarify the variability of reports on aluminium loading during hemodialysis. Recent work in the authors' laboratory (King et al., 1982; 1979) has been directed towards an attempt to separate aluminium species in plasma into more than just ultrafiltrable and protein-bound fractions. The approach used to define the plasma distribution and binding of aluminium was to employ gel filtration under equilibrium conditions which was a technique used previously in our laboratory for studying the distribution of calcium in plasma (Toffaletti et al., 1977).

Separations were carried out on a column packed with Sephacryl S-200 superfine with a column eluent containing per liter 140 mmol Na(I), 1.1 mmol Ca(II), 0.5 mmol Mg(II), 4.0 mmol K(I), and 10 mmol 2-[tris(hydroxymethyl)-methyl]aminoethanesulfonic acid

(TES) adjusted to pH 7.40 at 37°C. This eluting solution was analyzed, and the aluminium concentration was found to be 11 μg/L.

The typical elution profile for a patient on renal dialysis was similar to the pattern obtained in a normal subject (King et al., 1979). The first aluminium peak (Y) which eluted before peak A was not present in the serum from the normal volunteer (King et al., 1979).

The aluminium in the peak Y which created a species large enough to be excluded from the column must be complexed in some manner. Such a complex might be with protein, lipoprotein, cholesterol, or triglycerides. The fractions containing the aluminium in this peak were analyzed for cholesterol and triglyceride in order to determine if any lipid material containing these compounds was present, but none was detected. However, this observation could have been due to the relatively low sensitivity of the methods used. Since aluminium is capable of forming colloids, the aluminium may have been complexed in a colloidal species which would be excluded from the column and thus elute in the void volume. Neither the composition of the aluminium nor the significance of the aluminium in peak Y is understood. However, this peak has been present in the elution profile of 4 renal patients and has not been seen in the profile using serum from a normal volunteer.

Aluminium in peak A was associated with some high molecular weight proteins present in the early elution of protein peak 1. This peak has been previously shown to contain alpha-2-macroglobulin, IgM, haptoglobin, and some orosomucoid (Toffaletti et al., 1977). The present study has not provided information as to whether all of these proteins, a single one, or an undetected protein provides the binding of aluminium.

The aluminium in peak B was eluted in association with the albumin in peak III, and this amount of aluminium was greater in dialysis patients than in normal volunteer (King et al., 1979), undoubtedly due to the higher aluminium concentration in the serum of the dialysis patients. The two largest aluminium peaks were C and D.

The aluminium in peak C was probably bound to small inorganic species, such as phosphate and hydrocarbonate, which were reported to elute just before the potassium peak in a calcium-binding study using the same gel filtration technique employed here (Toffaletti et al., 1977). However, the aluminium also might have been associated with the protein material in this peak, as shown by absorbance at 280 nm. The aluminium in peak D was associated with a group of proteins and/or small polypeptides, as indicated by peak IV. Using polyacrylamide gel electrophoresis, two proteins were found under this peak with apparent molecular weights of 60,000 and 80,000. These proteins must have appeared in the later column fractions because of retardation owing to some interaction with the Sephacryl S-200 gel. Since amylase is known to interact with these type gels, the fractions were analyzed for amylase activity, and none was found. At this time, the identities of these two proteins are not known.

COLLECTION AND CONTAMINATION PROBLEMS

Types of Specimens

Many types of biological specimens can be analyzed for aluminium. Tissue has been suggested as the specimen of choice to evaluate the true body store of aluminium (Alfrey et al., 1976; Crapper et al., 1976; Maloney et al., 1982), and brain, bone and muscle have all been used (Alfrey et al., 1976; Crapper et al., 1976). Since these specimens are not readily obtainable on a regular basis, blood aluminium levels have been analyzed to reflect a patient's aluminium content (Gorsky and Dietz, 1978; Alderman and Gitelman, 1980; Smeyers-Verbeke et al., 1980; Gardiner et al., 1981; Oster, 1981; Leung and Henderson, 1982; Parkinson et al., 1982; Bertholf et al., 1983; Bettinelli et al., 1985; Brown et al., 1984). This analysis involves no digestion or ashing steps for sample preparation as found in tissue assays, thus eliminating a possible source of contamination. Most of these methods use serum as the specimen of choice instead of plasma, which eliminates the need to obtain an aluminium-free anticoagulant, a potential source of aluminium contamination. Plasma also has a greater tendency to form fibrin clots than does serum, requiring the insertion of some object into the specimen to remove the fibrin, thus introducing another possible source of contamination.

Urine has been analyzed for content with specimens being collected over a 24-hour period into plastic containers (Gorsky and Dietz, 1978; Smeyers-Verbeke et al., 1980; Leung and Henderson, 1982; Kaehny et al., 1977).

Samples of dialysate solution may also be analyzed (Kaehny et al., 1977; Branger et al., 1980; Pierides and Frohnert, 1981). Analysis on this type of specimen can help to evaluate commercial preparations of dialysate for contamination by aluminium. The effectiveness of a dialysis system can be evaluated by taking aliquots of dialysate solution during dialysis and analyzing for aluminium (Branger et al., 1980; Pierides and Frohnert, 1981).

Water samples from various sources can also be analyzed (Parkinson et al., 1982) such as tap-water from patients on home dialysis. Water used to prepare dialysate solutions should be checked as a possible source for introducing aluminium into the dialysis system. Aluminium-free water for the preparation of standards, solutions and dilutions for analysis can be achieved by distilling, deionizing, passing through reverse osmosis, or a combination of the three. Analysis of this type of water should result in no detectable levels of aluminium (Smeyers-Verbeke et al., 1980).

Balance studies involving aluminium make it necessary to perform fecal analysis. A recent report for the authors' laboratory describe methods of collection, shipping and analysis of these types of specimens (Brown et al., manuscript in preparation).

Sample Collection

Blood

Sample collection is, potentially, a major source of possible contamination. Needles, glass vacuum tubes, plastic syringes, anticoagulants, plastic tubes and other equipment used in standard venipuncture methods are all suspect for sources of contaminated aluminium. Each piece must be checked for a aluminium contribution to the specimen. Smeyers-Verbeke et al. (1980) checked plastic syringes used for blood collection by filling the syringe with Al-free water and allowing to sit overnight before assaying for aluminium. Parkinson et al. (1982) compared absorption signals between fresh samples and those samples held in the sampling container for a period of time. Other authors report finding spurious contamination in glass tubes, syringes, and plastic tubes (Alderman and Gitelman, 1980; Gardiner et al., 1981; Oster, 1981) while some report negligible amounts of aluminium found in their blood collection procedures (Leung and Henderson, 1982; Bertholf et al., 1983). Each laboratory should check all sources for possible contamination. Several methods are available for the preparation of aluminium-free plastic and glassware. Rinsing the glassware in several changes of a 5% (w/v) solution of nitric acid, or allowing the items to soak in the solution overnight, is one procedure that can be used. The items should then be rinsed in copious amounts of Al-free water (Gorsky and Dietz, 1978; Smeyers-Verbeke et al., 1980; Oster, 1981; Parkinson et al., 1982). Oster (1981) utilized a solution of nitric acid in Triton X-100. A saturated solution of disodium EDTA is useful in rinsing plastic tubes and other plastic ware (Alfrey et al., 1976; Leung and Henderson, 1982). This method eliminates the storage and handling of concentrated acids.

Stainless steel needles and plastic catheters used in blood collection have been reported to contribute negligible amounts of aluminium to the procedure (Gorsky and Dietz, 1978; Alderman and Gitelman, 1980; Gardiner et al., 1981; Oster, 1981; Leung and Henderson, 1982; Bertholf et al., 1983). The type of tube used for collection and its pretreatment, if any, is not widely agreed upon. Gardiner et al. (1981), Gorsky and Dietz (1978), and Parkinson et al. (1982) collected blood into a plastic syringe. The blood was then dispensed into an acid washed plastic tube. Oster (1981) allowed blood to drip from the end of the inserted needle into a pre-rinsed plastic tube. Alderman and Gitelman (1980), Leung and Henderson (1982), and Bertholf et al. (1983) collected blood directly into a glass vacuum tube, without anticoagulant or serum separator, or pre-rinsed. Kaehny et al. (1977) collected blood directly into a vacuum tube containing heparin. By looking at each author's reported normal range (Table 2), one can readily see there is essentially no difference between the ranges.

Gardiner et al. (1981) collected blood from normal subjects by two methods to determine the necessity for using elaborate collection procedures for drawing blood. In 19 subjects, a plastic cannula and syringe were used to draw blood. The blood was then dispensed into an acid-washed plastic tube. In 15 subjects, blood was collected using a stainless steel needle and syringe. The blood was then dispensed into a plastic tube that had not been acid-washed. The mean value found from each set of data were found to be

TABLE 2

SERUM ALUMINIUM VALUES

Reference	Collection Technique	Reference Range (μg/L)		
Gorsky and Dietz (1978)	Syringe/plastic tube	12	-	46
Parkinson et al. (1982)	Syringe/plastic tube	2	-	15
Oster (1981)	Plastic tube	2.5	-	10.0
Alderman and Gitelman (1980)	Plain glass vacuum tube	0	-	7.6
Leung and Henderson (1982)	Plain glass vacuum tube	2	-	14
Bertholf et al. (1983)	Plain glass vacuum tube	1	-	12
Kaehny et al. (1977)	Heparinized vacuum tube	2	-	12

not statistically significant from each other. These studies suggest that there is no need for elaborate collection methods if standard techniques produce the same results.

The blood collection procedure that has been in use in the authors' laboratory involves drawing blood with a stainless steel needle into a plain glass vacuum tube (No. 6430, Becton-Dickinson and Co., Rutherford, NJ 07070). The blood is allowed to clot approximately 20 min before centrifugation. Serum is transferred to a 17 x 100 mm polypropene tube (Falcon, Oxnard, CA). In establishing a reference range for healthy individuals, blood specimens from 50 subjects were collected by this method with a resulting aluminium range of 1 to 12 μg/L.

Tissue

Bone samples for aluminium analysis have been taken from the iliac crest at the time of biopsy or at autopsy (Alfrey et al., 1976; Maloney et al., 1982) and the specimen placed in an Al-free plastic container. Bone for histological staining is fixed in 10% buffered formalin (Maloney et al., 1982). Crapper et al. (1976) analyzed brain samples from specific areas of the cerebral cortex and from subcortical area. Alfrey et al. (1976) analyzed brain samples from frontal cortex. Whole brain as well as white and grey matter were analyzed. A description of how the specimen was handled before analysis was not provided. Crapper et al. (1976) transported and stored brain samples frozen in Al-free plastic containers and performed dissection from the frozen specimen in a dust-controlled room. All instruments and gloves were rinsed in aluminium-free water. At frequent intervals, this entire procedure was performed on standard homogenized freeze-dried brain powder to ensure little or negligible aluminium contamination.

Urine and feces

The present authors have developed procedures for collection and storage of urine and fecal specimens as follows: 24 h urine specimens are collected in plastic containers (Scientific Products, McGraw Park, IL) and the total volume is recorded. A 10 mL aliquot is transferred to a polypropene tube (Falcon, Oxnard, CA) which is stored at 4°C.

Fecal specimens are collected directly into plastic bags which are weighed at the end of a 24-h time period, placed in a paper container and frozen.

Transport and Storage

Blood drawn into glass vacuum tubes should be separated within one hour after blood is collected into a plastic tube. Variable results may occur if blood is allowed to remain in contact with glass (Bertholf et al., 1983). The cap should be tight fitting, or covered with parafilm. If the tube is to be shipped, the tube's cap must be wrapped in such a way as to prevent leakage upon handling. There is no indication in the literature that storing the specimen at room temperature results in a loss of aluminium as compared to storing the sample at 4°C. Specimens for aluminium analysis have been received in the authors' laboratory both at room temperature and on ice packs, and some normal samples when shipped at room temperature through the U.S. mail gave aluminium values all within our reference range.

Tissue samples should be placed into Al-free plastic containers and brain specimens especially need to be frozen until analysis (Crapper et al., 1976). Bone can be kept at room temperature.

Specimen collection for aluminium analysis has the potential of contributing varying amounts of the element into the specimen. Each step of the collection procedure must be scrutinized to determine if it is contributing any contaminating aluminium to the sample. Each laboratory should check all materials before collecting patient specimens. Once the collection procedure is established, scheduled checks of the procedure should be employed to verify little or negligible contamination is being contributed by the technique. A quality control check for the blood collection technique could involve drawing blood from healthy individuals once a week. The individual chosen should be in the same area as patients who are being drawn for aluminium levels. This method would enable monitoring of the sample collection procedure and transport for any spurious aluminium contamination. An aluminium value within the reference range would be acceptable for this specimen. Tissue samples from healthy, non-diseased persons at autopsy can serve as controls for bone, brain and muscle aluminium determinations.

Urine and fecal specimens can be shipped frozen on dry ice. Urines are then stored at 4°C, but for convenience, and aesthetic reasons, feces are sealed into 32 oz. plastic containers (Cole-Palmer, Chicago, IL) and kept frozen until processing.

Sources of Contamination in Analysis

As in specimen collection, every item used during analysis should be regarded as a potential source of aluminium contamination. Glassware, pipet tips, plastic tubes, sample cups, the working environment and the water utilized must all be checked to ensure that they are adding negligible amounts of aluminium to the procedure. The room chosen for the analyses should have a limited access to ensure that dust in the working environment is being circulated as little as possible. Sample preparation should be carried out in an environmental laminar flow hood; this precaution helps to minimize contamination by dust particles.

Water utilized for standard curve preparation, rinsing of glassware and sample dilutions must be of high purity. The water should produce a resistivity of at least 18 megaOhms; this characteristic should be continuously monitored.

All glassware should be made aluminium-free which can be accomplished using either by acid solutions or by saturated disodium EDTA. The acids used should be of ultra-pure quality. The method used in our laboratory is based on the dilute acid cleaning technique described by Brown et al. (1984).

Contamination is a major problem encountered in the performance of aluminium analyses. All items utilized during collection, transport, and assay should be checked for unwanted aluminium contribution to the procedure. Only by taking these stringent precautions will one be able to produce results with confidence in their accuracy.

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Chapter 14

Arsenic

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INTRODUCTION

In order to understand the role of arsenic in the environment it is necessary to have some knowledge of the chemical forms of arsenic present in different environmental media as well as their concentrations, biotransformation and interaction with biologically important molecules. This necessitates the availability of reliable methods for the speciation and quantification of arsenic in air, soil, water, and biological media.

Arsenic (atomic number 33, atomic mass 74.9216) is the 20th most abundant element in the earth's crust. It belongs to the elements of the 'P' block of the Periodic System where it is placed below phosphorus and above antimony. The mass numbers of its isotopes range from 68 to 80; however, only the natural isotope 75 is stable. The gamma-emitting radioisotopes ^{76}As (half-life 26.4 h), ^{74}As (half-life 17.77 d), and ^{73}As (half-life 80.3 d) are commercially available and often used for method development and control (Krivan, 1987; Krivan and Arpadjan, 1989). Elemental arsenic exists at room temperature as metallic or gray arsenic, and yellow arsenic. As a center element of the 'P' block it can be found both in metallic and covalent compounds. The oxidation states are -III, 0, +III, and +V. Arsenic trihydride (arsine, AsH_3) is a colourless, very poisonous, neutral gas with a characteristic garlic odour.

Arsenic is present in igneous and sedimentary rocks and ores mainly in the form of sulphides, arsenides, and sulpharsenides. Weathering, volcanism, dissolution in water, biological and anthropogenic activities lead to arsenic emissions into the atmosphere, the terrestrial and aquatic environment.

Normally arsenic levels in soils and sediment are on average 6 mg/kg (e.g. Riedel and Eikmann, 1986; Dudka and Markert, 1992), but may be much higher, particularly in soils in the vicinity of smelteries, and other industries using arsenic as well as in agricultural soils where pesticides, herbicides and defoliants have been used (see below).

Arsenic occurs naturally in water, mainly as arsenite and arsenate, but methylated forms may be present as a result of biological activity. Anthropogenic sources of arsenic are e.g. mining and preparation of fossil and mineral resources (arsenic sulphides), combustion of fossil fuels (mainly arsenic oxides), non-ferrous smelteries (arsenic trioxide), production of glass (arsenic trioxide), production of semiconductors (gallium arsenide), use of feed additives for poultry and pigs (arsinilic acid), production and use of arsenical pesticides (lead arsenate, calcium arsenate, arsenic acid, copper aceto-arsenite, sodium arsenite, methylarsonic acid, dimethyl arsinic acid), preservation of wood (ammoniacal copper arsenate and chromated copper and zinc arsenate), and cutting and burning of preserved wood. In some countries arsenic is still used as a medicine, e.g. sodium methylarsinate, and substituted phenylarsonic acids. (WHO, 1981, Woolson et al., 1983; Andreae, 1986; Léonard, 1991)

Table 1 shows the formulae of some commonly occurring arsenic compounds.

TABLE 1

CHEMICAL FORMULAE OF A SELECTION OF COMMONLY OCCURRING ARSENIC COMPOUNDS

Arsenic trioxide (arsenous oxide)	As_2O_3 (or As_2O_6)
Arsenite	AsO_3^{3-}, AsO_2^-
Arsenate	AsO_4^{3-}, $HAsO_4^{2-}$, $H_2AsO_4^-$
Arsenic trisulfide	As_2S_3
Gallium arsenide	GaAs
Methylarsonic acid (MMA)	$CH_3AsO(OH)_2$
Dimethylarsinic acid (DMA)	$(CH_3)_2AsO(OH)$
Arsenobetaine	$(CH_3)_3As^+CH_2COO^-$
Arsenocholine	$(CH_3)_3As^+CH_2CH_2OH$ X^-
Arsine	AsH_3
Trimethylarsine	$(CH_3)_3As$
Trimethylarsine oxide	$(CH_3)_3As=O$

ENVIRONMENTAL AND TOXICOLOGICAL SIGNIFICANCE

Most of the arsenic compounds that enter the environment undergo chemical transformation. With the exception of the arsenite-arsenate conversion, facilitated by the redox potential of the surrounding media, the transformation reactions are biochemically

mediated. The biomethylation of arsenic was recognized some time ago when arsines with a garlic-like odour were produced from cultures of a certain fungus (Challenger, 1945). In sea water algae play an important role in the reduction and methylation of arsenate, which is the predominant form in sea water (Andreae, 1977). Demethylation of methylated arsenicals may also occur (Sanders, 1979; Anderson and Bruland, 1991)

In addition to inorganic arsenic and methylated arsenic acids, methylarsenic-containing ribofuranoside derivatives may occur in algae e.g. the brown kelp *Ecklonia radiata* (Edmonds and Francesconi, 1981, 1983). Anaerobic decomposition of *ecklonia* converts the arsenic-furanoside derivatives to 2-dimethyloxarsylethanol (Edmonds et al., 1982), which can easily be metabolized further to the, from the present state of knowledge, practically nontoxic compound arsenobetaine, which is the main form of arsenic in most species of fish and crustaceans (Cannon et al., 1981; Edmonds and Francesconi 1987, 1988; Ballin et al., 1992). Dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO) and trimethylaarsine (TMA) have also been found in seafood (Norin et al., 1985; Edmonds and Francesconi, 1987; Whitfield, 1988; Arbouine and Wilson, 1992). Besides these water-soluble organo-arsenicals, marine organisms have been shown to contain small amounts of lipid-soluble arsenic compounds. (e.g.Irgolic et al., 1977; Shinagawa et al., 1983). Two compounds of this type have recently been identified by the HPLC ICP-MS examination of lipid materials rendered water-soluble by hydrolysis (Edmonds et al., 1992).

Arsenic speciation in the environment, with particular emphasis on marine and aquatic systems, has been comprehensively reviewed recently (Cullen and Reimer, 1989).

By far the most toxic compounds of arsenic are the inorganic forms. The fatal human dose for ingested arsenic(III)oxide has been reported to be in the range of 70-180 mg (WHO, 1981). Animal data indicate that As(III) is more toxic than As(V). Thus toxicity decreases in the following order: As(III) < As(V) < MMA (monomethyl arsonic acid) < DMA (dimethylarsinic acid) < arsenobetaine. Major symptoms following acute intoxication from inorganic arsenic are gastrointestinal effects, muscular cramps, facial oedema and cardiac abnormalities etc. Long-term exposure to inorganic arsenic through inhalation or ingestion can lead to a number of adverse health effects, including lung cancer (in the case of inhalation of arsenic), and effects on the liver, the cardiovascular system, the hematopietic system and the nervous system (WHO, 1981; Fowler, 1983; EPA, 1984; Arnold, 1988).

In the eighties a technical panel of EPA reviewed several studies on arsenic as a possible essential element in human diet. Since inconsistent results were found in animal studies and many uncertainties remained, the panel determined "that at present, the case for arsenic essentiality is not proven for animals, and is even less certain for humans" (Gostomski, 1987).

BIOTRANSFORMATION AND EXCRETION IN MAMMALS AND MAN

Inorganic arsenic is also methylated *in vivo* in mammals. Arsenate is rapidly reduced to arsenite, which is afterwards partly methylated (Vahter and Envall, 1983; Vahter and

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INGESTION                        EXCRETION
As(III)                          As(III)
                                 DMA
                                 MMA
As(V)                            As(V)
                                 DMA
                                 MMA
                                 As(III)
ARSENOBETAINE                    ARSENOBETAINE
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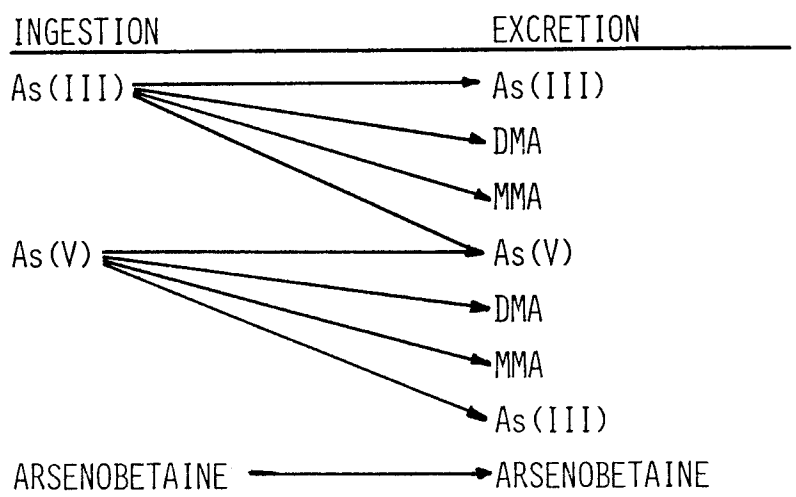
Fig. 1. Metabolism of arsenic compounds in mammals; note: at normal ingestion rates more than 60% of inorganic arsenic is transformed into DMA.

Marafante, 1985), see Fig. 1. In most animals studied dimethylarsinic acid is the main metabolite (Bertolero et al., 1981; Charbonneau et al., 1979, 1980; Vahter, 1981; Vahter and Marafante, 1988), while in man the urinary excretion consists under normal conditions - i.e. without excessive ingestion of inorganic arsenic - of about 20% inorganic arsenic, 20% MMA and 60% DMA (Buchet et al., 1980, 1981a; Smith et al., 1977; Tam et al. 1979). Methylation is to be considered as a detoxification of the inorganic arsenic, since the methylated metabolites are less reactive with tissue constituents and more easily excreted in the urine (Buchet et al., 1981a; Tam et al., 1982; Vahter et al., 1983, 1984). Absorbed MMA is further methylated to DMA, while dimethylarsinic acid is excreted mainly in an unchanged form (Buchet et al., 1981; Vahter et al., 1984). Only about 5% of the dose is methylated further and excreted in the urine as trimethyarsine oxide (Marafante et al., 1987). Arsenocholine is partly oxidized to arsenobetaine in vivo, while arsenobetaine is absorbed and excreted unchanged (Vahter et al., 1983). Studies with radioactively labelled (^{74}As) arsenate in man showed that e.g. 38 % of the dose was excreted in the urine within 48 hours and 58 % within five days (Tam et al., 1979). In subjects who ingested 500 mg As in the form of arsenite, 33% of the dose was excreted in the urine within 48 hours and 45% within 4 days (Buchet et al, 1981a). It is estimated that about 60-70 % of the daily ingested inorganic arsenic is excreted in the urine (Buchet et al., 1981b). The excretion of organic arsenic from fish, however, is significantly faster as was shown in a comparative excretion study (Burow and Stoeppler, 1987). About 76 % of the organic arsenic ingested with flounder (10 mg) was excreted in the urine within 8 days (Tam et al., 1982).

HUMAN EXPOSURE AND DOSE INDICATORS

Human exposure to arsenic has been mainly assessed in the past by the determination of the total arsenic concentration in blood, hair, nails, and urine. In cases of suspected arsenic poisoning gastric juice has also been analyzed. In order to obtain meaningful data, however, it is important to determine the chemical form of arsenic, at least in body fluids. Depending on the source and length of exposure, these indicators have a different meaning and different significance as well (Foá et al., 1987; Vahter, 1988).

Blood

Clearance of absorbed inorganic and organic arsenic is fairly rapid in man, i.e. arsenic levels in blood are elevated only for a short time following absorption. If exposure is continuous (e.g. through drinking water) blood arsenic may reach a steady-state and may then reflect the degree of exposure. This, however, is only the case if relatively high amounts of arsenic are ingested. At present no valid routine method for the specific determination of metabolites of inorganic arsenic in blood has been reported.

Hair

Arsenic concentrations in hair and nails are higher than in other organs because of the high keratin content. SH-groups of keratin are able to bind arsenic(III). Since it was shown that methylated metabolites of inorganic arsenic and arsenobetaine are not accumulated in hair (Vahter, 1988), arsenic in hair reflects exposure to inorganic arsenic only and is thus also a good indicator in cases of short-term exposure (by ingestion) i.e. also for arsenic poisoning. In these cases the determination of small hair sections along the hair may give reliable information on the time of acute exposure, or poisoning in forensic cases (e.g. Valkovic, 1977; Cross et al, 1979). A disadvantage of hair analysis is the well known fact that, especially in occupational exposure, it is almost impossible to differentiate between endogenous (intake) and exogenous (contamination) sources (Valkovic, 1977; Hopps, 1977; Chittleborough, 1980).

Also arsenic in water used for washing the hair may be bound and give rise to elevated levels.

Nails

For nails the situation is similar to that for hair. Arsenic might be deposited in the nail roots from the blood stream and then migrates distally as the nails grow. The risk of exogenous influences, of course, is also similar and probably even more pronounced.

Urine

Since arsenic is mainly excreted via the kidneys (see also section Biotransformation and Excretion) the determination of its concentration in urine is by far the predominantly used method to estimate exposure.

Arsenobetaine is the main form of arsenic which is taken up by man via the marine food chain in rather high amounts. Through the ingestion of a single meal of fish and/or crustaceans that may contain several mg arsenic in the form of arsenobetaine, which is rapidly excreted in urine (Burow and Stoeppler, 1987; Vahter et al., 1983), total arsenic concentrations can be found with levels sometimes well above 1 mg/L (Burow and Stoeppler, 1987; Norin and Vahter, 1981). The concentration of metabolites of inorganic arsenic in human urine is normally $<20\,\mu g$ As/L , while in persons occupationally exposed to inorganic arsenic the urinary concentration of inorganic arsenic metabolites may reach some 100 μg As/L (see Table 2). Thus measurements of total urinary arsenic alone may lead to a serious overestimation of the exposure to inorganic arsenic. This points to the fact that the speciation of arsenic in urine is of the utmost importance for valuable data as was shown in comprehensive metabolic schemes (Marafante and Vahter, 1989).

ARSENIC LEVELS IN BODY FLUIDS AND TISSUES

Table 2 presents data for typical levels of inorganic arsenic and its metabolites and total arsenic in various excreta and tissues of normal and exposed subjects based on a selection of scientific papers. Forensic cases, not included in the table were addressed by Solomons and Walls (1983) and by Cross et al. (1979). Since information is still limited and has many uncertainties, most data from exposure are given as an upper limit for general information only.

Urine

The table includes data for arsenic in the urine of nonexposed subjects (e.g. Smith et al., 1977; Buchet et al., 1980; Norin and Vahter, 1981; Schierling et al., 1982; Valkonen et al., 1983; Apel and Stoeppler, 1983; Stoeppler and Apel, 1984; Vahter and Lind, 1986; Foà et al., 1987; Jensen et al., 1991, Sabbioni et al., 1992). Due to some intake of inorganic arsenic from marine food (seaweed) the inorganic arsenic in urine in Japan was reported to be somewhat elevated compared to data from Europe (Yamauchi and Yamamura, 1979).

Data are also included for inorganic arsenic and its metabolites in urine following occupational and environmental (drinking water) exposure (e.g. Morse et al., 1979; Yamamura and Yamauchi, 1980; Cant and Legendre, 1982; Schierling et al., 1982; Takahashi et al., 1983; Vahter et al., 1986, Abdelghani et al. 1986; Franzblau and Lilis, 1989).

TABLE 2

TYPICAL VALUES FOR TOTAL(T) OR INORGANIC ARSENIC AND ITS METABOLITES(I) IN BODY FLUIDS AND ORGANS; VALUES ARE EITHER μg/L FOR FLUIDS OR μg/kg (DRY WEIGHT) FOR SOLID MATRICES

Matrix	Normal levels	Occup. exposure (Drinking water)
Urine(I)	< 2-20 (50)	up to $4 \cdot 10^3$
Whole blood(T)	< 0.5-4	up to 300
Blood serum(T)	< 2-7	
Cerebrospinal fluid(T)	< 0.5	
Head hair(I)	< 10^3	up to $7 \cdot 10^5$
Pubic hair(I)	< 10^3	up to $4 \cdot 10^5$
Finger nails(I)	< 10^3	up to $8 \cdot 10^5$
Toe nails(I)		up to $3 \cdot 10^5$
Stomach(T)	< 100	
Kidney cortex(T)	< 50	up to 200
medulla	< 50	
Liver(T)	< 60	up to 200
Heart(T)	< 40	
Lung(T)	< 100	up to 10^3
Lymph nodes(T)	< 60	
Aorta(T)	< 150	
Bone(T)	< 10	< 200

Blood, serum or plasma, and cerebrospinal fluid

Data of normal (total) arsenic levels in blood (Table 2) are taken from earlier and more recent studies (e.g. Heydorn, 1970; Bencko and Symon, 1977; Foà et al., 1987; Burow, unpublished, Sabbioni et al., 1992) as well as those obtained after exposure to arsenic from drinking water (Heydorn, 1970; Valentine et al., 1979; Foà et al., 1987). Values for (total) arsenic in serum are taken from a study with neutron activation analysis (Damsgaard et al. 1973) with direct determination by graphite furnace AAS (Pegon, 1985) and in a second-generation biological reference material using neutron activation analysis and hydride generation atomic absorption spectrometry (Versieck et al., 1988). Values for total arsenic in cerebrospinal fluid are taken from a recent study (Sabbioni et al., 1992).

Hair, nails, organs, and bone

Normal levels of arsenic in hair, nails, organs and bone were taken from several basic studies (e.g. Smith, 1964; Liebscher and Smith 1968; Hopps, 1977; Lindh et al., 1980; Brune et al., 1980; Aalbers et al. 1987, Sabbioni et al., 1992). The studies for kidney, liver,

lung, and bone also include some data for occupationally exposed workers (Brune et al., 1980; Lindh et al., 1980).

ANALYTICAL CONSIDERATIONS

An analytical procedure consists of proper sampling, sample storage, if necessary, sample preparation, which is different for total elemental analysis and species analysis, separation procedures, if speciation or preconcentration is required, the quantification step, and quality assurance. All this applies to arsenic analysis and thus will be treated subsequently and demonstrated by practical examples for all these steps. Since speciation for arsenic is of paramount importance, it will be described in some detail.

Sampling and sample storage

Though arsenic occurs in the order of several mg/kg in street and house dust (Fergusson and Kim, 1991) and it has been shown by radio release tests (after reactor irradiation of the used steel needles) that there is some arsenic on disposable stainless steel needles (0.8 μg/L) and in the washers and cylinders of syringes (approx. 3 μg/L) (Minoia et al., 1992) careful cleaning of all used tools, glass and plastic ware as described in detail in the Chapter on Sampling and Sample Storage. of this book, will usually minimize the risk of contamination for whole blood and urine to an acceptably low level.

In a very detailed interlaboratory study, recently published and also of general importance for the sampling and storage of urine (Schaller et al., 1991b), it was stated that urine should be collected in plastic bottles, acidified with acetic acid (1 mL per 100 mL urine) and stored in the refrigerator or deep frozen until the samples are taken for analysis. This procedure was also reported to be sufficient for the preservation of arsenic species in urine for the routine methods described below that differentiate between total inorganic arsenic metabolites and fish-derived arsenic (i.e. mainly arsenobetaine) only. If the urine has to be divided into several aliquots it should be homogenized. This is of particular importance if the urine has been stored at low temperatures. To achieve valid results, any sediment, which is already present or may have occurred during storage, should be dissolved by warming. If this is not completely successful the remaining sediment should be distributed as homogeneously as possible by careful mixing before withdrawing the aliquots to be analyzed. It is recommended that urine specimens which have been stored at low temperature be warmed in a shaker water bath before aliquotation. After homogenization, the specimens should be allowed to cool down to room temperature. Other authors just proposed the addition of hydrochloric acid (1mL per 100 mL of urine) and storage in polyethene bottles at 4 °C for preservation (Norin and Vahter, 1981).

Dissection of organs with normal arsenic levels for refrigerated storage and/or the subsequent analysis should be performed under strictly contamination-controlled working conditions with pre-cleaned knives and appropriate tools made from quartz, plastics, or titanium, preferentially on clean benches. These restrictions, of course, are not necessary

in poisoning or intoxication cases, where a normal laboratory environment usually suffices.

Sample preparation for total arsenic determination

Except for the direct analysis of arsenic in body fluids by atomic spectroscopic methods or neutron activation analysis, and cold acid solubilization of solid samples (containing mainly inorganic arsenic) followed by hydride AAS (Haswell et al., 1988), most methods for total arsenic determination require a complete decomposition of all arsenic compounds present. This can be achieved by a number of dry and wet decomposition procedures that are amply described in the literature (see also the Chapter on Sample Treatment of this book). Thus only examples of the most frequently and successfully applied approaches for subsequent arsenic determination are given below.

Dry ashing

An effective method for dry ashing prior to total arsenic determination was first published some time ago (Uthe et al., 1974) and successfully applied in a somewhat modified form later (e.g. Norin and Vahter, 1981; Tam and Lacroix, 1982; Vahter and Lind, 1986). It consists of the addition of a certain amount of a mixture of $Mg(NO_3)_2 \cdot 6H_2O$ dissolved in water with addition of MgO to the solid or liquid sample. Usually 10 mL ashing aid is added to sample amounts of 1-10 g in a 150 mL beaker and mixed well. The beaker is covered with a watch glass and dried with its contents at 110 °C in an oven. After this, it is transferred into a cold muffle furnace, the temperature slowly raised to 500 °C and maintained overnight. Then, the sample is cooled to room temperature, distilled water added, and the mixture is dissolved in 6 M HCl. For the subsequent determination of arsenic, the addition of 1 mL of a 30% KI solution (reaction time 15-20 min) is recommended in order to reduce As(V) to As(III) prior to hydride AAS.

An elegant alternative is combustion in an oxygen stream using the Trace-0-Mat, a partially mechanized apparatus (Kürner Analysentechnik, Rosenheim, FRG). The sample is ignited and the volatile trace elements, including arsenic, are condensed on a cold finger filled with liquid nitrogen. Subsequent refluxing with nitric acid in a quartz test tube below the combustion chamber collects both the volatilized elements from the previously cooled areas and nonvolatile elements in the residual ash (Knapp et al., 1981). Procedure: approx. 0.2 g of sample is placed into a little cup made of ash-free filter paper (e.g. Schleicher & Schüll No 589), which is fixed in the quartz sample holder of the Trace-0-Mat. The apparatus is closed, and the cold finger is filled with nitrogen. The IR radiators are focused on the upper edge of the filter paper cups to ensure slow combustion of the sample after ignition. A stream of oxygen (70mL/min) is started simultaneously with the ignition. After about one min the sample is completely ashed. The residual liquid nitrogen is then removed from the cold finger, and the IR radiators focused on the nitric acid in the test tube. After about 30 min of boiling under reflux, the apparatus is allowed to cool and

the remaining nitric acid solution containing all the arsenic present as As(V) can be used for subsequent hydride AAS determination (Welz and Melcher, 1985).

Wet ashing

Because of the resistance of e.g. arsenobetaine to wet and pressurized decomposition with nitric acid, higher temperatures and more effective acids are required for complete decomposition of materials that contain resistant arsenic compounds. For years this was accomplished with mixtures of nitric and perchloric acids or mixtures of nitric, perchloric and sulphuric acids (Pershagen et al., 1982). For example, urine was decomposed by pipetting 2 mL of the sample into test tubes (18x150mm) held in an aluminium heating block (Peter et al., 1979). The digesting acid (1:1 mixture of concentrated nitric and perchloric acid) was added and the heating block was kept on a hot plate at approx. 200 °C for 4-6 h until approx. 0.5 mL liquid remained. This liquid was then used after appropriate dilution for arsenic determination by hydride AAS.

Human organs were similarly decomposed by the use of a mixture of nitric (8 mL) and perchloric (2 mL) acid on a sand bath until a clear, colourless solution was obtained. This solution was then allowed to evaporate to fumes of perchloric acid and subsequently appropriately diluted with hydrochloric acid (Subramanian and Méranger, 1982).

More elaborate and safer decomposition has been performed in the following manner. Approx. 0.1 g of dry matter, 1 mL of deionized water, and 1 mL of nitric acid (65% w/v) are transferred into 40 mL quartz flasks with a long neck placed in an appropriate aluminium block with holes for these flasks, heated slowly to 140 °C and kept at this temperature for 30 min. After the solution is cooled to room temperature, 0.5 mL of sulphuric acid (96% w/v) and 0.2 mL of perchloric acid (70% w/v) are added. The temperature is then slowly raised over 105 min to 310 °C and kept at this temperature until the solution has evaporated to about 0.5 to 1 mL, which takes about 15-20 min. After the solution has cooled to room temperature, 20 ml of 5 M HCl is added, and the solution is heated to about 90 °C for another 10 min. After cooling, the solution is diluted to an appropriate volume with deionized water. Arsenic is obtained in the pentavalent state (Welz and Melcher, 1985). Digestion with nitric, sulphuric and perchloric acids can be routinely performed in various materials by the use of programmable automatic digestion systems as well (e.g. Burow and Stoeppler, 1987).

A similar procedure, evaluated for the determination of selenium in body fluids by hydride AAS, has been successfully used for arsenic as well. The flasks are the same as already described above with a long neck and 40 mL volume. However, flasks of similar design (e.g. Kjeldahl flasks) may be used as well. The procedure is as follows. 0.5 mL serum, whole blood or urine is placed into the digestion flasks. If necessary a sample volume of 1.0 ml may be also used. After this 1 mL of nitric acid (65% w/v) is added, the digestion flask placed into the aluminium heating block, and the block slowly heated to 140 °C. This temperature is maintained for 25 min and then cooled to room temperature. 0.5 mL (96% w/v) sulphuric and 0.2 mL (70% w/v) perchloric acid is added to the cool solution. The subsequent programme consists of: slowly heating (approx. 15 min) to

140 °C and maintaining this temperature for 15 min, slowly increasing the temperature to 200 °C (approx. 10 min) and maintaining it for 15 min. The temperature is slowly increased to 250 °C (approx. 10 min) and maintained for 15 min, slowly increased to 310 °C (appprox. 10 min) and maintained for 20 min. The heating block is cooled to near ambient temperature. After the addition of 20 mL 5 M HCl solution, heating to 90 C and maintaining this temperature for 20 min the solution is cooled to room temperature, and can be diluted to volume and used for arsenic analysis (Welz et al., 1987).

As far as problems with the use of perchloric acid are concerned the authors stated that "the risk is minimal if the recommended decomposition procedure and acid volumes are used"

It should be mentioned, however, that in all work with perchloric acid special fumehoods are absolutely mandatory.

If mainly inorganic arsenic in hair, organs and body fluids has to be determined, e.g. in poisoning cases, less violent decomposition procedures, e.g. mixtures of nitric and sulphuric acid (Curatola et al., 1978), nitric acid, and nitric acid with hydrogen peroxide (Solomons and Walls, 1983) are sufficient.

Ashing in closed systems ("pressure ashing")

As already mentioned above, the application of open wet decomposition and even nitric acid pressure decomposition in PTFE vessels up to approximately 160-170 °C is not sufficient for the complete destruction of all arsenic compounds (Welz and Melchers, 1985). If, however, nitric acid is applied at higher temperatures using the so-called high pressure asher (HPA) and decomposition vessels made from quartz glass (Knapp, 1985, Würfels, 1989), which can reach reaction temperatures of up to 300 °C, all arsenic compounds are completely destroyed so that a reliable subsequent determination of total arsenic without any loss during thermal treatment is possible. This method avoids, in addition, the use of perchloric acid and is thus certainly a contribution to safer sample preparation. The relatively high cost of this system, however, has hitherto prevented it from being introduced into a larger number of laboratories.

The use of the HPA system is fairly simple: the 30 mL or the 70 mL standard quartz vessels allow intakes of sample equivalent to 100 or 230 mg carbon, respectively, with nitric acid (65 to 69% w/v) volumes of 2 mL per 100 mg C. The heating programme from ambient to maximum temperature is as follows: ambient to 250 °C: 1 min; 250 to 300 °C: 30 min. This maximum temperature is maintained for 90 min then turned off. After cooling (approx. 60 min) the samples are made up to volume and treated as required for subsequent determination. In one of the author's laboratory (M.S.) the analyte solutions are treated with a KI-ascorbic acid mixture for reduction to As(III) and subsequent FIA-AAS-determination with excellent precision and absolute detection limit (Guo et al., 1990).

Microwave systems with quartz glass decomposition vessels are now commercially available attaining short-term reaction temperatures well above 250 °C (Dunemann, 1991). Thus in the near future it might be possible to decompose difficult samples for total arsenic determination using this technique.

Sample preparation for subsequent speciation

Body fluids

Comprehensive chromatographic speciation procedures for arsenic compounds often have to be performed without any pretreatment in fresh urine (e.g. Norin and Vahter, 1981). If chromatography is applied after refrigerated storage in PTFE bottles, filtration through 0.45 μm (Chana and Smith, 1987; Sheppard et al., 1992) or even 0.2 μm membrane filters (Heitkemper et al., 1989) has been reported as well.

Solid materials

Although there have been only very few studies about arsenic speciation in organs, from the numerous procedures published for speciation of arsenic in food and marine organisms additionally some examples will be given here in order to provide an introduction to the sample preparation techniques also applicable to solid samples of human origin.

Because of the stability of most of the arsenic compounds of interest for speciation studies, nearly all work commences with homogenized, predominantly lyophylized, materials. Extraction of arsenic species from dry and finely ground samples is often performed with methanol/water/chloroform mixtures or just methanol (Luten et al., 1982; Francesconi et al., 1985; Momplaisir et al., 1991; Ballin et al., 1992). Methanol and phosphate buffer (1:1) extraction was applied with especially good results for methylated forms of inorganic arsenic (Arenas, 1991). Rather simple approaches are the solubilization of various types of organic materials by treatment with e.g. tetramethyl ammonium hydroxide (TMAH) at ambient temperature for the subsequent determination of inorganic arsenic and its metabolites (Stoeppler and Apel, 1984, Burow and Stoeppler, 1987) and the solubilization of As(III) and As(V) by treatment with perchloric acid and $Fe_2(SO_4)_3$ at elevated temperatures with subseqent separation and determination of As(III) and As(V) (Holak and Specchio, 1991). Some speciation procedures based on NaOH treatment will be described under Speciation procedures.

Determination of total arsenic

Total arsenic determination is necessary in many cases, particularly in intoxication, for balance studies, and for food control if only total arsenic contents are needed. However, it is only a complement to the various speciation procedures and the methods described in this section are very often used to determine the arsenic amount that occurs in a distinct chemical form or fraction as part of a so-called hyphenated procedure. As far as these methods are described in detail under Speciation procedures the final determination step will also be outlined.

Table 3 summarizes relative detection limits for the analytical methods mainly used at present, based on the arsenic concentration in a liquid analyte, in an analyte solution after

TABLE 3

RELATIVE DETECTION LIMITS FOR ARSENIC USING VARIOUS ANALYTICAL METHODS

The detection limit is defined as three times the standard deviation of the background noise or blank (3s) in the analyte solution or in a solid matrix. Values reported in the literature as 2s are converted to the more realistic 3s value. It should be mentioned, however, that the given figures have to be multiplied by at least a factor of 3 for the **determination limit** (see also text).

Method	Detection limit [μg/kg resp. μg/L]
ICP-AES[1]	20
Flame AAS[2]	30
Graphite furnace AAS with Zeeman background correction[3] (20 μL)	1
Hydride AAS (at least 20 mL)[2]	<0.03
Voltammetry[4]	0.2
ICP-MS[5]	0.04
Total reflection XRF(TXRF)[6] (50 μL)	0.2
Instrumental NAA (INAA)[7] (^{76}As)	0.05

Sources and remarks:
[1] (Boumans and Vrakking, 1987)
[2] (Welz, 1985)
[3] (Slavin et al., 1988)
[4] (Bodewig et al., 1982)
[5] (Hieftje and Vickers, 1989)
[6] (Michaelis, 1989)
[7] (Hoste, 1986; INAA data are based on a sample weight of 500 mg non interfering matrix and the following irradiation and counting conditions: Thermal neutron flux 1×10^{13} n cm^{-2} s^{-1}; irradition time 5h maximum; counting with a 40 cm Ge(Li) detector; sample-to-detector distance: 2 cm; zero decay time before start of count and maximum counting time: 100 min. Detection limit for arsenic can be improved, if radiochemical separation or group separation is applied after irradiation and sample decomposition (e.g. Samsahl et al., 1968; Tjioe et al., 1977)

appropriate decomposition procedures, or in solid material. Methodological details may be further found in a recent overview article (Irgolic, 1992).

The table only gives a rough (relative) picture of the potentialities of the various analytical methods. Practically attainable determination limits are often much higher because of the dilution of e.g. urine and blood samples frequently necessary, and the use of sample digests that introduce dilution factors of 10 and even higher.

Methodological examples

Neutron activation

From the given detection limits it is obvious that in the past neutron activation analysis (NAA) was the most promising method for basic studies (e.g. Heydorn, 1984). This is also reflected in the literature particularly for the quite low normal arsenic levels in human materials (see data and references for Table 2). Despite the fact that this method is less used at present routinely it still is valuable as a reference method, and particularly useful for the certification of reference materials as a multielement approach (Van Renterghem et al., 1992).

There is an interference with bromine in irradiated samples (e.g. urine) containing some bromine in that radioisotopes of bromine (^{82}Br) and arsenic(^{76}As) both have their prominent gamma-intensities at 0.56 MeV and very similar half lives (36 h and 26,7 h respectively). Thus it is necessary, if no chemical separation is performed prior to gamma counting, to subtract the contribution from ^{82}Br in the 0.55 MeV gamma peak (Norin, 1983).

For the determination of arsenic in biological material approx. 300 mg of dried matter was sealed in ultraclean silica vials, irradiated at a thermal neutron flux of approximately $9x10^{12}$n cm^{-2}sec^{-1}. After irradiation the vials were washed with concentrated nitric acid, frozen in liquid nitrogen to reduce the internal pressure and crushed in a plastic container. The samples were then decomposed under pressure with a mixture of concentrated nitric and sulphuric acid, evaporated to near dryness after opening the vessels, taken up in hydrochloric acidic solution, made up to volume and after reduction with KI subjected to an arsine generation step. The evolved arsines were trapped, the trap sealed into a polyethene vessel and the 560 keV gamma photopeak from ^{76}As counted. The authors reported absolute detection limits of either 0.5 μg or 0.05 μg depending on the detector used (Orvini and Delfanti, 1979)

In another study freeze-dried human material (up to 2g) was irradiated at a thermal neutron flux of approximately $2x10^{13}$n cm^{-2}s^{-1} for 3 days, then left to decay for three days and subsequently reactivated for 30 min (Brune et al., 1980). After this the material was decomposed with a mixture of concentrated sulphuric acid and hydrogen peroxide and various elements, arsenic included, distilled simultaneously according to a multielement separation scheme previously developed (Samsahl et al, 1968). Gamma-spectrometric measurements were then performed on the separated fractions. Arsenic contents as low as 1 μg/kg (wet weight) were easily determined.

Arsenic was determined in freeze-dried samples of human autopsy tissue (Aalbers et al., 1987) by gamma-counting following appropriate irradiation and absorption on an ion exchanger after a semiautomated separation procedure together with radionuclides from Se, Sb, and Hg (Tjioe et al, 1977). Arsenic contents as low as 1 μg/kg (dry weight) were easily determined.

A radiochemical procedure for the determination of twelve trace elements in human blood serum, including arsenic, after reactor irradiation at a thermal neutron flux of $4.3x10^{12}$ n cm^{-2} s^{-1} using the k_o standardization method was recently developed with the

aid of radiotracers of the elements to be determined. The procedure consisted of oxygen combustion of the irradiated sample (decay period 18 h) in the Trace-O-Mat system, subsequent absorption and fractional elution from a strong anion exchanger (DOWEX 1-X8). As, Cu, Fe(II) and Se were eluted and gamma-counted together. The procedure was successfully applied to the determination of all trace elements in a human blood serum reference material (Van Renterghem et al., 1992).

Voltammetry

A particular advantage of voltammetry for arsenic analysis is that direct determinations of As(III) in diluted urine are possible down to approx. 20 μg/L using the differential pulse mode with a quite inexpensive instrumentation that permits quick direct analyses in cases of intoxication or poisoning, provided the laboratory personnel has sufficient experience with this methodology. The analytical procedure starts with the dilution of 2 mL urine with 18 mL 1 M sulphuric acid, 0.1 mL 0.2 M disodium-EDTA solution and 0.2 g NaCl. The reference electrode is Ag/AgCl/cKCl = 3 mol/L and the working electrode a rotating disk electrode with a gold tip. Electrodeposition is performed after 15 min deaeration with nitrogen at -0.4 V for 300 s rotation time. After 30 s rest time the potential is scanned from -0.4 V to +0.5 V with a pulse amplitude of 50 mV, the potential change is 5 mV/s and the sensitivity 50 nA/mm. This method has been compared with AAS in a case of fatal arsenic poisoning and excellent agreement achieved (Daldrup, 1989).

Better sensitivity can be achieved if As(III) is extracted from hydrochloric solutions with e.g. benzene and the voltammetric determination of arsenic is performed either directly in the organic solution or after evaporation of the solvent and transformation into an aqueous solution (Gemmer-Colos, and Neeb, 1987).

Atomic absorption spectrometry

Atomic absorption spectrometry is nowadays certainly the most frequently used and most versatile method, also in combination with chromatographic techniques (see below). The hydride technique offers in addition also the potential for direct and exclusive determination of As(III) at pH values >4, which is of advantage for the rapid detection of arsenic poisoning in human biopsy and autopsy samples. Hydride techniques are now *being* used in various modes and with sometimes significantly decreased absolute detection limits with at the same time minimized interferences from other elements. This is particularly the case if preconcentration techniques - e.g. introduction of volatile hydrides into pre-heated graphite furnaces (Sturgeon et al., 1987) and cryogenic trapping (Arenas et al., 1988; Arenas, 1991) with absolute detection limits at the 20-50 pg level are applied. Further significant progress has been achieved by coupling flow injection and hydride AAS with absolute detection limits <20 pg, and a nearly complete freedom from interferences allowing the combination with a number of very effective separation procedures (e.g. Burow and Dürbeck, 1993; Tsalev et al., 1992a, 1992b; Guo et al., 1989)

Despite a somewhat inferior relative detection ability and matrix interferences, which, however, could be more and more overcome by recent improvements e.g. the use of palladium nitrate/magnesium nitrate modifiers and the so-called stabilizied temperature

platform furnace (STPF) concept, the graphite furnace has distinct advantages for the quick and reliable determination of total arsenic either directly in liquid and solid biological materials or in digests that need not necessarily be complete (Slavin, 1984, Schlemmer and Welz, 1986; Slavin et al., 1988).

Total arsenic in urine was determined by the graphite furnace with Zeeman effect background correction. Using matrix modification with 5% nickel nitrate, sample volumes of 20 μL, charring up to 1500 °C, atomisation at 2800 °C, and using the standard addition method was reported to achieve a detection limit of 10 μg/L if the urine dilution factor (2) was considered (Edgar and Lum, 1983).

In another method with Zeeman background correction as well, applying nickel and magnesium nitrate in nitric acid as matrix modifier, and the L'vov platform, charring up to 1400 °C, atomization at 2400 °C, the detection limit, based on a 1 + 7 dilution of the urine, was reported as about 8 μg/L in terms of the original undiluted urine (Paschal et al., 1986).

The direct determination of arsenic in blood serum was performed with the graphite furnace, L'vov platform and Zeeman background correction. The use of nickel (0.2 M) and 2% Triton X-100 was found to be the best approach. Charring was performed at 1600 °C, atomization at 2600 °C. A 1 + 1 dilution was sufficient and the detection limit found for this solution was 0.4 μg/L (Pegon, 1985).

The behaviour of As(III) and As(V) in a graphite furnace during the individual steps of the temperature programme was studied by use of the radiotracer [76]As for urine, human serum and hair solubilized with tetramethyl ammonium hydroxide (TMAH). Significant stabilization effects were observed if various metals, including nickel, were used as matrix modifiers. Thus experimental conditions for the determination of arsenic in urine, human serum and hair were optimized. For the determination of the hair solution, charring at 1200 °C and atomization at 2400 °C were found to be optimal (Krivan and Arpadjan, 1989).

Solid sampling graphite furnace AAS with an inner miniature cup was used for arsenic determinations at higher contents. Matrix modification with 60 μg Ni in a solution containing 3 M sulphuric and 4 M nitric acid was reported as necessary for reliable measurements. The results indicated acceptable bias at the mg/kg level (Atsuya et al., 1987).

Speciation procedures

On the one hand there are relatively simple procedures that allow a quick determination of the sum of inorganic arsenic, i.e. As(III) and As(V), MMA and DMA for epidemiological studies, occupational exposure, intoxication, and poisoning. On the other hand, more complex and detailed procedures are required for scientific investigations on arsenic metabolism in mammals and man.

Since in very many cases the differentiation between total arsenic, including fish-derived arsenobetaine and toxic forms of arsenic, is the most important task, this approach will be described in as much detail as possible and some of the other procedures are outlined and references given for the reader's further information.

Liquids

Quantification of inorganic arsenic and its metabolites (exclusively urine)

The fact that only As(III), As(V), MMA and DMA in urine can be transformed directly into volatile hydrides by the addition of sodium borohydride, while fish-derived arsenic such as arsenobetaine and arsenocholine does not react with this compound, was the basis for a quick and reliable hydride AAS procedure (Norin and Vahter, 1981). This procedure has been successfully applied in other laboratories as well (Schierling et al., 1982; Apel and Stoeppler, 1983; Stoeppler and Apel, 1984; Burow and Stoeppler, 1987).

However, recent studies have shown that ingestion of some types of seafood may give rise to arsenic species in urine, which do react with sodium borohydride (Vahter, 1986; Arbouine and Wilson, 1992; Mürer et al., 1992). Arbouine and Wilson (1992) reported that DMA was the main form of hydride forming arsenic compound in several fish species. Furthermore, seafood may also contain trimethylarsine oxide and trimethylarsine (Norin et al., 1985; Edmonds and Francesconi, 1987; Whitfield, 1988). Storage of fish samples at -20 °C increased the levels of TMAO, indicating degradation of arsenobetaine and/or arsenocholine (Norin et al., 1985). Therefore, it is recommended that the subjects under study should avoid eating seafood, or products containing seafood, for 2-3 days prior to urine collection.

In their basic work Norin and Vahter (1981) investigated carefully any interference from arsenic compounds of marine origin by the addition of synthetic arsenobetaine and arsenocholine to urine samples and by analyzing the urine of fish eaters. In addition separations and separate determinations were performed based on previous work by other authors for all four arsenic compounds using identical compounds as calibrants. Interferences from other elements were also studied and found not to be significant. It was also observed that, provided the peak area was measured, no difference in peak area occurred for the same arsenic amounts of As(III), As(V), MMA and DMA. Thus calibration could be simply performed with standard solutions prepared from sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$). In order to improve stability sodium borohydride solutions were passed through 0.45 μm membrane filters in accordance with a previous paper (Knechtel and Fraser, 1978).

The procedure started with acidified urine (1 mL HCl to 100 mL of urine) and used a commercially available mercury/hydride system (MHS-10, Perkin Elmer) attached to a Perkin-Elmer 360 AAS instrument with EDL (resonance line at 193.8 nm). The peak area measurements and peak recording were performed with a single-channel integrating recorder.

Subsamples of 0.5-5 mL of urine were analyzed directly without any pretreatment. If needed, deionized water was added to the urine to give a final volume of about 5 mL in the reaction chamber. The addition of 0.5 mL of HCl conc. decreased the pH to 1-1.5. After this sodium borohydride [4% in 0.05 M NaOH] was added for the arsine generation.

The same principle is the basis of a "Selected Method" of the "Deutsche Forschungsgemeinschaft", "Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area", recently published (Schaller et al., 1991b).

The description of this method provides some more information in addition to that given above. From practical experience it was of advantage to add antifoaming emulsion (e.g. Antifoam 110 A from Dow Corning) to the analyte solution in the reaction chamber in order to avoid occasional foaming in routine work. For daily use detailed pipetting schemes are also given for the preparation of the calibration standards from As_2O_5 for the standard addition method. In contrast to the results reported for the previous method (Norin and Vahter, 1981), the paper showed that within the observed error limits comparable results could be achieved either by peak height or peak area evaluation in various normal and spiked urines with As(V) as the calibrant. However, for cases of occupational exposure, containing elevated amounts of As(III), peak area evaluation is recommended. Detection limits for 1 mL of urine were reported as 1 μg/L for peak height and 2 μg/L for peak area evaluation.

A typical programme for the determination of total inorganic arsenic and metabolites is shown in Table 4 for a commercially available mercury/hydride system (MHS-20 from Perkin-Elmer).

Comprehensive tests were performed for between-day imprecision and accuracy with several participating laboratories. With normal contents long-term precision was around 6% and recovery rates were between 88.4 and 104.6 %.

Various arsenic compounds

Since the first report on various arsenic species in human urine (Braman and Foreback, 1973) numerous papers have appeared describing further studies and presenting improved and increasingly sophisticated procedures. Thus, some examples of these hyphenated procedures will be given in the following.

TABLE 4

PROGRAMME FOR THE DETERMINATION OF THE SUM OF INORGANIC ARSENIC, MMA AND DMA IN A HYDRIDE SYSTEM (SCHALLER et al., 1991b)

Analytical step	duration [s]
Purge (whole system) with inert gas –purified nitrogen or argon– (approx 1500 mL/min)	30
Reduce inert gas flow rate to ca 600 mL/min	
Flush reduction solution (0.5 mL/s) into the reaction chamber with a pressure of 150 hPa on the reduction solution vessel	12
Flush the generated hydrides into the heated quartz cuvette (1000 °C) with simultaneously generated hydrogen	
Purge (whole system) with inert gas (approx. 1500 mL/min)	30

The first procedure for the separation of As(III), As (V), MMA and DMA in natural waters and urine depended on pH-selective reduction reactions with sodium borohydride and separation of the volatile arsines produced by selective volatilization from a cold trap. Quantification was performed by atomic emission spectrometry (Braman and Foreback, 1973). This method was later modified on the basis of another approach for arsenic speciation in natural waters (Andreae, 1977): 0.5 to 5 mL of unpretreated urine is diluted with 50 mL of deionized water in the reaction chamber of a mercury-hydride system. The pH is then adjusted to 1-1.5 by addition of 5 mL of oxalic acid (10% w/v). Helium is subsequently passed through the system for about 1 min to remove the oxygen. After this 6mL of sodium borohydride (4% in 0.05 M NaOH) is added. The generated arsines are trapped in a U-tube immersed in liquid nitrogen. When the U-tube is removed from the liquid nitrogen, arsines of inorganic arsenic, MMA and DMA are volatilized successively due to their different boiling points and travel via the carrier gas into the AAS detector (Norin and Vahter, 1981).

Another, relatively simple method, also applicable to plasma samples, is as follows. Bio-Rad AG 50W-x8, 100-200 mesh cation exchange resin (7,5 g) was packed into a 10-mL disposable glass pipette (0.85 x 16.5 cm) and conditioned with 0.5 M HCl (Tam et al., 1979). One millilitre of plasma, urine or arsenic reference solution was applied to the top of the column. Then, the column was eluted successively with 0.5 M HCl (As(III) and As (V), deionized water (MMA), and first 5% (v/v), then 20% (v/v) ammonia (DMA). A modification of the elution procedure, using 0.5 M HCl, deionized water and 1.5 M per-chloric acid, has been reported by Marafante et al. (1987). Arsenic may be detected by gamma counting for radiolabelled arsenic or XRF or hydride AAS for nonradioactive arsenic compounds (Tam et al. 1979; Vahter, 1986). Separation of As(III) and As(V) in the HCl fractions has been performed using a weakly basic resin (AG3-X4A, 100-200 mesh, Bio Rad) (Vahter and Envall, 1983). The HCl-fractions were neutralized with NaOH and applied to the column. As (III) was eluted with 0.01 M phosphate buffer, pH 6.9, and As(V) with 1 M HCl.

In another method the methylarsenic acids were derivatized with thioglycolic acid methylester to yield lipophilic species for subsequent gas chromatography either using a flame ionization detector or the more selective thermionic detector (Beckermann, 1982). The method permitted the quick determination of methylarsenic acids in urine and blood of animals after arsenic ingestion. With a sample volume of 5 mL and extraction with 0.5 mL cyclohexane a determination limit of 10 mg/L was reported.

By combining a hydride cold trap system with gas chromatography with multiple-ion detection (GC-MID), inorganic arsenic and methylated forms were separated and determined (Odanaka et al., 1983) The detection limit was 0.2-0.4 μg/L for a 50 mL sample. The authors reported the detection of trimethylarsenic compounds in the urine of laboratory animals for the first time.

As(III), As(V), DMA and MMA were determined by an automated hydride AAS method after high-perfomance liquid chromatographic separation using an anion-exchange silica-based column (10-m Ionosphere with a conditioning column) in urines from occupationally

unexposed persons. (Ghana and Smith, 1987). The reported detection limits, corrected for 3s (see remarks in Table 3), are 3 μg As/L for all four species.

Single-column ion chromatography (Wescan Anion/R-IC) and subsequent detection by ICP-MS was used for the separation of As(III), As(V), DMA and MMA by elution with 2% propan-1-ol and gradient elution by 50 mmol/L carbonate buffer, pH 7.5 (Sheppard et al., 1992). The order of elution was: As(III), DMA. MMA, Cl⁻ and As(V). The mass spectral interferent ArCl$^+$ was reduced by chromatographically resolving chloride from the negatively charged arsenic species. Absolute detection limits differed for the analyzed species and ranged from 70 pg for DMA to 260 pg for As(V).

The last example also includes solid material and the analysis of arsonium compounds, i.e. arsenobetaine, arsenocholine, and tetramethylarsonium in seafood and human urine. Urine (5mL) was diluted with 50 mL of ethanol and placed in a dry ice-acetone bath for 20 min. The supernatant was separated from the resulting precipitate by centrifugation and flash evaporated. The residue of this solution was redissolved in 10 mL of water and applied to the head of an anion exchanger column of DOWEX 2X8 (0.5 x 10 cm). The column was washed with additional distilled water to result in a total volume of 30 mL of eluate. This eluate was acidified to pH 3 with HCl. The acidified aqueous solution was extracted four times with liquefied phenol. These extracts were combined and backwashed three times with water to remove excess chloride and salts. The phenol layer was diluted with 75 mL of diethyl ether, and evaporated to dryness. The residue was resolubilized in methanol and concentrated to 1 mL under a gentle stream of nitrogen. 50 μL aliquots were analyzed by HPLC-AAS. The column consisted of a cyanopropyl bonded-phase (5-μm silica support). The mobile phase delivered at 0.65 mL/min consisted of 80% (v/v) methanol, 19% diethyl ether and 1% (v/v) glacial acetic acid, containing 0.12% (v/v) triethylamine and 20 mg/100 mL picrosulphonic acid. The HPLC-AAS interface was all quartz and converted the arsenic compounds via a combustion chamber and the addition of hydrogen to their hydrides that were atomized in a cool microdiffusion flame. Recovery was reported to be between 85 and 97% and detection limits at the nanogram or sub-nanogram level (Blais et al., 1990; Momplaisir et al., 1991).

Solid samples

An example is the procedure given above for arsonium compounds. Another approach for this type of compounds is the extraction with methanol/water/chloroform. Based on this pretreatment two procedures will be briefly summarized.

Arsenic(III), As(V), MMA, DMA and arsenobetaine were separated by HPLC and determined on-line by ICP-MS. Two forms of HPLC were used: ion pairing and ion exchange, with absolute detection limits for arsenic in the range from 50 to 300 pg independent of the arsenic species when peak area evaluation was used. Anion pairing was found to be more sensitive to changes in the analyte matrix. Anion exchange was more tolerant because of the higher buffering capacity of the mobile phase while cation pairing was found suitable for the determination of DMA and arsenobetaine in a biological sample containing high concentrations of salts (Beauchemin et al., 1988, 1989).

The basis of the second fairly simple routine method is transformation of arsenobetaine and arsenocholine into trimethylarsine and determination by headspace gas chromatography with a flame ionization or AAS detector. A detection limit below 10 μg/kg is attainable under optimized working conditions (Ballin et al., 1992; Ballin, 1992).

If lyophilized material is extracted with a methanol/phospate buffer solution and appropriate conditions are chosen the separation of arsenobetaine from MMA, DMA and inorganic arsenic might also be easily possible in human organs with an acceptable yield and detection limits at low μg/kg levels (Arenas, 1991)

The solubilization of biological material with TMAH at least permits the determination of As(III) in human biopsy and autopsy materials by a pH-dependent reaction (pH 5) with sodium borohydride and also the determination of total inorganic arsenic by reaction at pH values around 1-1.5; for this method, however, the use of antifoaming solution is always mandatory (Stoeppler and Apel, 1984).

A similar approach for arsenic speciation in solid samples is dissolution in e.g. 2 M NaOH. This was performed for various tissue and organs deep frozen stored before analysis (Yamauchi and Yamamura, 1983) as well as in hair and urine (Yamato, 1988). The samples are heated at 95 °C and the obtained mixture diluted with water prior to analysis. MMA, DMA and TMA are not decomposed by this treatment so that the species, including inorganic arsenic, may be separated and determined by liquid nitrogen trapping-hydride AAS. The same approach, using 3 M NaOH was also applied to seafood (Mohri et al., 1990). Arsenobetaine is decomposed to trimethylarsine (see also the procedure of Ballin, 1992), while MMA and DMA were not decomposed. Thus speciation by liquid nitrogen trapping-hydride AAS may be performed subsequently as well.

Arsenic in solid biological samples was speciated by a pyrolysis-NAA-procedure developed with radiochemically labelled arsenic compounds (Gallorini et al., 1987). The method involved addition of NaOH and initial pyrolytical treatment of the sample in a quartz boat up to 380 °C in an inert atmosphere. Under these conditions inorganic arsenic remains in the boat and organic arsenic is nearly quantitatively volatilized and trapped on a cation exchange resin (AG 50X4) in plastic columns. These columns were subsequently irradiated in a Triga Mark II reactor (5×10^{12} n cm^{-2} sec^{-1}) and gamma counted for quantification.

QUALITY ASSURANCE

Interlaboratory comparison studies have been reported on the analysis of total arsenic in urine in Canada (Savoie and Weber, 1983; Weber, 1986). Control samples were prepared by pooling urine samples from subjects ingesting seafood or As(III) or by spiking urine samples with DMA or As(V). In the 1983-year study the average deviation from a "target value" (not further specified) was ± 30%. In the 1985-year study about 60% of the participating laboratories (about 40) reported results within 15% or 25 μg/L of the "target value".

The results of an interlaboratory comparison programme operated since 1979 for several toxic substances in blood and urine by the Centre de Toxicologie de Québec, Canada are summarized by Weber (1988). For urine arsenic, as for the other substances an increase of the number of participants with time could be seen. For arsenic in urine the relative standard deviation was concentration dependent and was lower for easily digested species (As(III), As(V)) than for resistant ones (e.g. seafood As). The success rate was approximately 60-70% for the inorganic arsenic and 40% for that of seafood origin. No chronological trend was apparent for the former but a slight downward trend could be seen for the latter.

Scattering results with "seafood arsenic", probably due to decomposition problems, are also obvious from intercalibrations of trace metals in (marine) biological tissue organized by the International Council for the Exploration of the Sea (e.g. Berman and Boyko, 1992).

In order to assess the overall performance of toxicological laboratories in the European Community (EC) and the applicability of analytical methods a long-term interlaboratory survey of the quality of (total) urinary arsenic and thallium was performed with twelve laboratories representing ten member countries (de Zeeuw et al., 1987). The results of the individual laboratories as well as the interlaboratory data showed in general an acceptable performance for bias and precision.

In Germany, due to a technical rule for dangerous agents (TRGS 410), issued by the Ministry of Labour in 1989, toxicological analyses in biological materials must be carried out under conditions of "statistical quality control". This QC scheme provides internal and external control programmes. For internal QC, the results of many years of experience with five commercially available control specimens and one "home-made" control have been evaluated recently. The control samples showed good, comparable results over a long period. Except in a few cases, there was good agreement with the assigned values. In addition, since 1982, the German Society of Occupational Medicine has offered eight intercomparison programmes for external QC. In samples from 80-90 laboratories, six metals in blood and around 20 inorganic, including arsenic, and organic parameters have been analysed. Successful participation was certified if both results obtained for one parameter were within the tolerance range (assigned value ± 3 SD). The average success rate was around 60% (Schaller et al., 1990, 1991a). The evaluation of the recommended method for total inorganic arsenic in urine included some interlaboratory comparison studies using normal and spiked urines, also including DMA. The results based on this method showed an acceptable variation of around 20% (Schaller et al. 1991b).

Human urine samples spiked with As(V), MMA and DMA at three different concentration levels have been prepared and used for quality control purposes in studies on the concentrations of arsenic metabolites in urine (Vahter and Lind, 1986; Vahter et al., 1986).

Certified reference materials are commercially available for total arsenic concentrations in e.g. water, bovine liver, some plant materials, various environmental materials, oyster tissue, albacore tuna, mixed diets, milk powder and urine (LGC, 1992) but no materials are yet available with certified amounts of arsenic species. However, work is under way at

BCR, Brussels for the preparation and certification of As(III), As(V), MMA, DMA, arsenocholine and arsenobetaine in a first selection of real samples (BCR, 1992).

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Chapter 15

Cadmium

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INTRODUCTION

Cadmium is a soft, bluish-white metal which is easily cut with a knife. It is similar in many respects to zinc. The melting point is 320.9 °C, the boiling point 765 °C (Weast, 1986).

The element belongs to the large group of transition elements or d block. The position within the Periodic Table of the elements is below zinc and above mercury. The zinc group has a filled d^{10} orbital and is transitional between the d block and the p block elements of boron and others. The outer electronic configuration of the zinc group is $d^{10}s^2$ and the common oxidation state is + II.

Besides the stable + II oxidation state, Cd may also form very unstable oxidation state + I compounds. Cd has a relatively low standard potential of -0.402 V (for M^{2+} + 2 e = M), which resembles more that of zinc (-0.762 V) than that of mercury (+0.854 V). This low standard potential reflects the reducing power of Cd.

Cd(II) resembles magnesium and many of the compounds are isomorphous. CdF_2 is stable to water and poorly soluble. The chlorides, bromides and iodides are 10-30 times more soluble in water than the fluorides. CdS and CdO are insoluble in water. Cd forms four-coordinated and six-coordinated complexes which are relatively stable. It also forms organic compounds of the type R_2Cd (Mackay and Mackay, 1968).

TOXICOLOGICAL AND ENVIRONMENTAL SIGNIFICANCE

Distribution

Cadmium commonly occurs in isomorphic form in zinc minerals such as zinc blende (ZnS) with Cd contents of 0.1-0.5%, and galmei ($ZnCO_3$) with Cd contents up to 5% (Stoeppler, 1991).

The average Cd content of the earth's crust is estimated to be about 0.1 mg/kg. Weathering of minerals in geological periods has led to Cd enrichment of sediments by a factor 2-3. Phosphates show a broad range of Cd contents with an average of about 15 mg/kg (Stoeppler, 1991). Soils contain generally < 5 mg/kg: it is stated that higher concentrations reflect anthropogenic inputs. Unpolluted waters contain very small amounts of Cd and values less than 1 μg/kg have been reported (Thornton, 1992).

World production and consumption of Cd has continued to rise over the past decade, with a production of about 17,000 tonnes/year in 1989 and a consumption of 18,000 tonnes/year. Industrial and mining operations are responsible for the release of Cd in the atmosphere (WHO, 1992a).

Cd has been emitted in greatly increased quantities after 1945 in the form of dusts and aerosols into the atmosphere, effluent into freshwater, and as solids from anthropogenic industrial activities (Stoeppler, 1991). Cd has a relatively high vapor pressure. The vapor is oxidized quickly to produce cadmium oxide in the air. When reactive gases or vapor, such as carbon dioxide, water vapor, sulfur dioxide, sulfur trioxide or hydrogen chloride, are present, the vapor reacts to produce cadmium carbonate, hydroxide, sulfite, sulfate or chloride, respectively. These salts may be formed in stacks and emitted into the environment (WHO, 1992b).

Some of the cadmium salts, such as the sulfide, carbonate or oxide, are practically insoluble in water. However, these compounds can be converted to water-soluble salts in nature under the influence of oxygen and acids; the sulfate, nitrate, and halogenates are soluble in water as mentioned before (WHO, 1992b).

Natural occurrence of Cd in atmosphere is mainly due to volcanic activity. In sea water upwelling leads to elevated levels in surface waters. The Cd level in sedimentary rocks may be considerably higher than 0.1 mg/kg, and thus can be transported by rivers to the oceans (WHO, 1992b).

Anthropogenic activities of protective plating of metals, polyvinyl chloride stabilizers, pigments, Ni-Cd batteries, and alloying are the most important, with a diminishing share of pigment and electroplating and a growing share of Ni-Cd batteries (WHO, 1992b).

Dissolved species are extremely labile, absorb less than other trace metals to reactive particles, and are released first from sediments and suspended particles, once the chemical conditions of the aquatic system change (Muntau and Baudo, 1992).

Bioaccumulation

Several years ago it was concluded by Eisler (1985), that some trends could be observed regarding the Cd residue bioaccumulation, i.e :
— Cd residues in plants are generally less than 1 mg/kg, but depending on Cd content and pH of the soil this may be considerably higher
— marine organisms generally contain higher Cd residues than freshwater or terrestrial species
— accumulation within vertebrates into the kidney and liver
— positive relation with age

- higher levels in animals living near urban and industrial sources, but not in sea birds and sea mammals
- season of collection, ambient Cd level, and sex of the organism may affect the Cd residue level

Cultured lettuce and chard on acid or calcareous soils to which Cd sulfate had been added, led to lettuce leaf concentrations of up to 800 mg/kg and chard leaves up to 1.6 g/kg in acid soil. In calcareous soils the levels were much lower. The same trend has been reported for rice, soybeans, ryegrass, and winter rape. Some invertebrates can concentrate Cd, as has been reported for earthworms, terrestrial snails, and slugs. In birds an accumulation into the kidney and liver have been found. Oysters or mussels can be used to monitor Cd in sea water levels, because a concentration of up to 100 mg/kg have been found after 100 hours (WHO, 1992b).

Toxicity in the environment

Cd is toxic for many plants and invertebrates, e.g. bacteria, algae, protozoa, duckweeds, floating ferns, sea urchins, sand dollars, starfishes, mussels, oysters, clams, bay scallops, oyster drills, mud snails, garden snails, ragworms, copepodes, amphipodes, shrimps, sandworms, crabs, squids, isopodes, scuds, daphnia's, nematodes, collembolan, mites, crayfish, mayflies and stoneflies.

Toxicity for fish, amphibian, birds, and wild small mammals has been well established (WHO, 1992b).

HUMAN EXPOSURE AND DOSE INDICATORS

Numerous publications in journals and books exist on this aspect. Cadmium is, together with lead, the element most studied regarding human toxicology. Therefore, only some interesting points will be given. For overviews about this subject the reader is referred to recently published general literature, i.e. Stoeppler and Piscator, 1988; Glaser, 1990; Merian, 1991; Nordberg et al., 1992; WHO 1992a; WHO ,1992b; Seiler et al., 1993.

Distribution in man

The daily intake of Cd in humans obviously depends on the personal diet. It is observed that the daily intake of Cd increases by about 3 μg for each consumed handpeeled shrimp containing brown meat (Elinder, 1992). In 'uncontaminated' areas the average daily intake is reported to be in the range of 10-60 μg/day for a person of 70 kg. Total daily intake of Cd in Germany (Müller, 1993), in the United States, in most other European countries and in New Zealand is very likely in the 10-30 μg/day range Schelenz, 1983; Elinder, 1985; Herber, 1993). In rural 'uncontaminated' areas in Japan the daily Cd intake has been estimated at 59-113 μg, whereas in contaminated areas in Japan average daily intakes as high as 400 μg have been reported (Friberg et al., 1986).

Contamination of drinking water may occur as a result of a low pH, especially in combination with Cd-containing silver solders of fittings and pipes, water heaters and coolers, and taps. Also drinking water from wells where the soil has been acidified by acid rain can contain higher Cd amounts (Friberg et al., 1986).

Cd in air occurs in particulate form, and thus is in fact a dry aerosol, comparable with smoke of a cigarette. It is clear from this example that the distribution of a dry aerosol in a volume of air will be far less homogeneous than in case of a wet aerosol or gas. Moreover, a tendency to deposition occurs, which depends on the particle size. Cd-A can thus be measured directly as the concentration in air or as deposit (Herber, 1993).

The presence of Cd in floordust presents a possible risk to young children who accidentally ingest dust by hand to mouth activity (Thornton, 1992).

The most important lifestyle factor regarding Cd intake is tobacco smoking, especially cigarettes and self-rolled cigarettes. Smoking of one cigarette, generally containing 1-2 μg of Cd, results in the inhalation of about 0.1-0.2 μg Cd (Friberg et al., 1986). Higher intake of Cd via smoking leads to a higher Cd-B concentration; it was reported that for each cigarette smoked per day the level of Cd-B increases 1.6% (Wibowo et al., 1982).

In the working environment levels up to 0.5 mg/m^3 in battery factories, but generally not higher than 100 μg/m^3 are reported (Friberg et al., 1986).

Toxicokinetics

The absorption of inhaled Cd in air is 10-50%; the absorption of Cd in cigarette smoke also 10-50%. Absorption is dependent of particle size and type of compound; respirable particles of a diameter below 5 μm and water soluble particles will probably absorb the best.

Gastro-intestinal absorption is estimated to be 5%. In a duplicate basket study it was found that daily intake was about 15 μg Cd for 24 h (Herber, 1993). In this study it was concluded that about 1-2% of the daily meals sampled had a Cd content that exceeded the tolerable level proposed by WHO.

Absorption of Cd is higher in females than in males, due to differences in iron stores (Lauwereys, 1978). Transport of Cd in the intestinal tract is influenced by the presence of various food components such as proteins and amino acids. The proteins L-cystein and L-histidin, which form relatively stable complexes with Cd, increase the transport (Herber, 1993). Whole-body retention of Cd was estimated as being 20-30% 3-5 days after administration, yet the intestinal uptake was calculated as 6%, only (Andersen et al., 1992).

Excretion of Cd via the faeces is about 90%; thus Cd-F can be used as an estimation of oral Cd intake and approximates the uptake by gastro-intestinal absorption (Herber, 1993).

Of the several models developed in the literature to describe the toxicokinetics of Cd, an eight compartment model as developed by Nordberg and Kjellström (1979) seems to fit best. This model uses three different blood compartments, one for whole blood, one for Cd accumulation in erythrocytes and one for Cd bound to metallothionein or to other SH groups of proteins. The Cd in the first blood compartment may exchange with different

tissue compartments. The binding of Cd in the tissues appears to be much stronger than in the blood compartment, therefore the transfer from blood to the tissues should be more efficient than vice versa.

It is often assumed that the kidney and liver represent at least 50% of the total body burden (Herber et al., 1988). With a Cd in the liver level below 40 mg/kg the Cd in the kidney level seems to increase with increasing Cd in liver. Cd levels in the kidney above 40 mg/kg Cd in the liver however, will decrease with increasing Cd in liver levels; here the kidney might lose Cd and this will be excreted in urine (Ellis et al., 1981). In a later *in vivo* study Ellis et al. (1984) concluded that most workers with a Cd in liver concentration above 70 mg/kg were judged to have some evidence of kidney abnormalities.

The highest average value of tissue burden related to age was found at 40-49 years of age in the kidney with a level of 4.9 mg, and liver (1.8 mg), all w.w. of whole organs (Salmela et al., 1983).

Toxic effects

In the past, exposure to high concentrations of fumes, appearing from heated Cd metal or compounds has lead to acute poisoning and in some cases to the death of workers. Principal symptoms reported were respiratory distress due to chemical pneumonitis and oedema. Cd-A values measured were 50 mg/m^3 during 1 h, or in another case, 8.6 mg/m^3 during 5 h. It has been estimated that an 8 h exposure to 5 mg Cd/m^3 will be lethal (WHO, 1992a).

In the past (1940-1950) cases of acute food poisoning were described, but nowadays acute poisoning by ingestion, aside from accidents, are rare. Recovery from acute poisoning appears to be rapid and complete (WHO, 1992a).

The critical organ for chronic Cd exposure is the kidney. In case of short-term peak exposures of workers, the lung may be the critical organ (WHO, 1992a).

Kidney dysfunction is one of the most characteristic signs of exposure to Cd (WHO, 1992a). The renal dysfunction induced by Cd is tubular, but claims have been made that Cd can also cause primary glomerular disease (Lauwereys et al., 1974; Bernard et al., 1976, and 1979), but this remains controversial (Piscator, 1988). In the working environment at high exposure levels, workers have developed proteinuria, renal glucosuria, aminoaciduria, hypercalciuria, phosphaturia, and polyuria. Signs of distal tubular damage are observed, and in a few severe cases, the renal damage progresses to a reduction in glomerular filtration (WHO, 1992a). Since in 1950 the first observations were made that proteins appeared in the urine of Cd workers (Friberg, 1950), proteinuria has proved to involve proteins with a molecular mass of 10,000 to 40,000 Daltons and is called *tubular proteinuria* (WHO, 1992a). Tubular proteinuria has been observed in numerous studies both in the working and in the general environment. It has been stated that Cd induced renal lesions are not reversible (Roels et al., 1982; Piscator, 1984). In Japanese Cd-polluted areas, signs of renal dysfunction very similar to those in workers have been found. Proteinuria and glucosuria were found to be common among the exposed people in one area (WHO, 1992a).

At present the most sensitive indicators of effect are β_2M-U and RBP-U, both belonging to the low molecular mass proteins (Herber et al., 1988). Enhancement of β_2M-U started at a Cd-U concentration of 3-6 μg/L (Piscator, 1984; Verschoor et al., 1987). Where low molecular mass proteins are used for the assessment of tubular damage, the determination of RBP-U is to be preferred over β_2M-U, as the latter protein already deteriorates in the bladder at pH 5.5. For the determination of β_2M-U, RBP-U and also Alb-U a standardized latex immunoassay method is available (Herber et al., in press). Another possibility to assess tubular proteinuria and glomerular proteinuria is SDS-Page electrophoresis (Herber et al., 1988).

Determination of tubular enzymes as NAG, β-galactosidase, β-glucuronidase, angiotensine converting enzyme, and gamma glutamyl transpeptidase in urine is another way of assessing of tubular damage. It appears that NAG is the next in sensitivity after RBP. Determination of β_2M-S or Alb-U is possible to assess glomerular damage (Herber et al., 1988). Also IgG-U is determined for this purpose.

None of the mentioned renal effect parameters are specific for Cd. Other nephrotoxic agents such as mercury, chromium, lead, organic solvents and pesticides may also induce changes into these parameters (Herber et al., 1988). Table 1 gives an overview of the most common tubular and glomerular proteins, and tubular enzymes connected with Cd.

Chronic inflammation of the nose, pharynx, and larynx have been reported in Cd workers in the past. Anosmia is a frequent symptom in Cd workers after prolonged exposure (WHO, 1992a).

Chronic obstructive lung disease of varying degrees of severity is frequently seen in Cd workers. Symptoms were connected with dyspnoea and emphysema, rather than bronchitis. The mortality increases in case of lung effects (WHO,1992a).

TABLE 1

LEVELS OF SOME PROTEINS AND ENZYMES IN SERUM AND URINE (after Herber et al., 1988)

Compound	Medium	Healthy people, not occupational exposed
α_1-microglobulin	urine	1-10 mg/L
β_2-microglobulin	urine	50-300 μg/L
β_2-microglobulin	serum	1.4-2.3 mg/L
retinol binding protein	urine	20-200 μg/L
total protein	urine	< 250 mg/L
albumin	urine	1-12 mg/L
NAG	urine	2-7.5 MU/L
immunoglobulin G	urine	1-8 mg/L

In several studies a relation between Cd exposure and blood pressure is assumed. Most human studies, however are hampered by the fact, that confounding variables such as smoking habits, and in environmental studies, other air pollutants, makes it difficult to draw solid conclusions about this effect (WHO, 1992a).

Increased mortality from lung cancer has been observed in several occupational cohorts exposed to Cd, and there is some evidence of dose-response relationships in two of the examined populations. Case control studies did not give support for such a relationship. It is difficult to reach a firm conclusion about causality, because a lot of confounding variables such as tobacco smoking and co-exposure to other compounds existed.

Investigations of the relationships between Cd exposure and prostatic cancer are inconclusive (WHO, 1992a). Also other studies regarding cancer in the working environment are still inconclusive, mostly due to the many confounders, e.g. co-exposure by other elements as arsenic and nickel (Boffeta, 1992; Kazantzis et al., 1992; Stayner et al., 1992; Boffeta et al., 1992).

CADMIUM LEVELS IN BODY FLUIDS

Regarding Cd levels in the different body compartments literature data have to be considered with great care. First, concentrations around the μg/L level are in principle prone to contamination during sampling and determination (see section 'Quality Assurance'). Secondly, a lot of parameters may influence the levels in especially blood and urine, e.g. a linear relation exist between the number of cigarettes smoked and the Cd in blood concentration (Wibowo et al., 1982). An international group of experts (TRACY) are judging publications in journals on the suitability for establishing reference intervals. In case of Cd the group started with Cd in blood and results will be published into two publications (Alessio et al., in press; Herber et al., in preparation).

Cadmium in blood

Cd in blood may be used as a biological monitoring measure for recent exposure in occupation or general environment.

The results of the earlier mentioned TRACY group for Cd in blood can be mentioned shortly as follows:

— in the majority of the publications no sufficient information was available about the analysis procedure to use the data of these publications for reference intervals. Often no quality control or control of contamination were mentioned

— parameters influencing the Cd in blood concentration are smoking of cigarettes, occupation, gender, area, and country; thus in fact a stratification has to be made for all these classes. Some data extracted from these publications are mentioned in Table 2. In case of smokers the concentration of Cd in blood may be assessed by a linear regression calculation of this level using the number of cigarettes as the independent variable.

TABLE 2

SOME TYPICAL LEVELS OF CADMIUM IN BLOOD OF HEALTHY, NON EXPOSED NON-SMOKERS IN DIFFERENT COUNTRIES

Non smokers, geometric means (levels in μg/L)	Country	Reference
0.19	Sweden	Friberg and Vahter, 1983
0.64	China	ibid
0.30	Mexico	ibid
1.06	Japan	ibid
0.52	U.S.A	ibid
0.38-0.53	Germany	Brockhaus et al., 1983
1.15-1.38	China	Watanabe, 1989a
1.58	Korea	Watanabe, 1989b
1.78-2.22	Japan	ibid

Cadmium in urine

Cd in urine may be used as a biological monitoring measure for the body burden. At low exposure levels Cd in urine reflects the total accumulation of Cd in the body.

Also in case of Cd in urine, quality control is of utmost importance. Moreover as the risk of contamination of urine by dust from clothing can be severe, the procedure of avoiding contamination must be mentioned in papers to judge the reliability of the data given.

Smoking is not an important parameter, but age is positively related with Cd in urine. Other parameters influencing the Cd level are gender, occupation, area and country.

TABLE 3

SOME TYPICAL LEVELS OF CADMIUM IN URINE OF HEALTHY, NON EXPOSED HUMANS

Level, Geometric means	Age group	Country	Reference
< 2 mg/kg creatinine	< 65 years	Germany	Ewers et al., 1985
< 1 mg/kg creatinine	30-69 years	Netherlands	Herber, 1992
< 1 μg/L	27-46	Sweden	Vahter et al., 1992
0.76-0.99 μg/24 h	adjusted for age (50 years)	Belgium	Sartor et al., 1992

A common problem in urine levels is the dilution. In case of Cd in urine, levels generally are given corrected for creatinine or urinary density.

Some reliable data are given in Table 3.

Cadmium in hair

The determination of Cd in hair does not present reliable information on the exposure, body burden or oral intake (Wibowo et al., 1986; WHO, 1992a). Ranges may be as wide as 0.2-110 mg/kg (Herber et al., 1983; Wibowo et al., 1986; WHO 1992a).

Cadmium in faeces

The determination of Cd in faeces may be used to assess the intake of Cd by ingestion or via the lungs. A reliable level is given by Vahter et al., 1992 , i.e. 5-12 μg/day (women 27-46 years old)

Cadmium in tissues

Critical levels has been suggested for Cd in kidney cortex of 50 mg/kg wet weight (Brown et al., 1978), but other authors put this up to 200 mg/kg.

For the liver a critical range from 20 to 25 mg/kg wet weight has been suggested (Herber et al., 1988)

ANALYTICAL CONSIDERATIONS

Determination of cadmium in blood

Considering the requirements of detection limit and contamination-free sample handling, determination of Cd-B with graphite furnace atomic absorption spectrometry (GFAAS) is the method of choice. In an international intercomparison program for the determination of Cd-B, GFAAS was far the most used (Herber et al., 1990a). This was also concluded in reviews about Cd determination (Stoeppler, 1984,1986). The detection limit of this most widely used method is about 0.04 μg/L (Herber et al., 1990a). This enables the determination of the lowest concentrations reported of 0.1 μg/L (see also Table 2). A standardized method for the determination of Cd-B will be mentioned here, including sample preparation. The method for Cd-B consists on deproteinization with nitric acid, followed by direct determination (Stoeppler and Brandt, 1980) and has been described more comprehensively by Herber et al. (1990a).

Pre-analytical sample preparation

The laboratory staff has to be informed about the health hazards in manipulating blood samples of patients who might be carriers of hepatitis, HIV and other viral diseases.

In case of collection of samples for occupational health studies collection must take place separately from the production sources of Cd. Preferably collection must take place within the occupational health service in a separate building or room not contaminated with Cd. Contamination is likely from work clothes. Clothes, overalls etc used during labour must be changed in a separate room before entering the sample collection room. Especially when voiding urine, contamination is likely from work clothes.

In case of environmental health studies, collection must take place within a local health service not contaminated with Cd.

Equipment and cleaning procedures

Use cleaned plastic collection tubes with stoppers. The whole set (tube **and** stopper) must be tested to deliver less than **10%** extraneous amounts of cadmium. Do not use coloured stoppers. Do not use plastics with a Cd-softener. Before starting the study test 6 test tubes per tube production lot. During the study frequently test blank tubes, e.g. for **1%** of the total number of samples to study. Test may be performed with a 1% v/v solution of nitric acid (e.g. Suprapur quality) in bidistilled water. Preferably use vacuum collection tubes. No special type of needles is required. Needles of stainless steel are adequate. Other needles, e.g. siliconized or iridium needles may be used as well but are not necessary. Use vinyl gloves, free of talc. Use cleaned plastic test tubes. See further collection tubes. Do not use glassware.

Blood collection

In case of a venipuncture clean the antecubital fossa of the arm with bidistilled water and analytical grade ethanol (the latter without cotton, just a rinse) and allow to dry by evaporation. Venipuncture is done with a needle. In case of a finger prick use the extremes of one of the fingers. Clean carefully twice with bidistilled water and twice with analytical grade ethanol (the latter without cotton, just rinse) and allow to dry by evaporation. It must be remarked that in case of a finger prick the risk on contamination is considerably higher than in case of a venipuncture. Finger pricks should be performed only when venipuncture is not feasible. All personnel handling blood must wear gloves. Use polyethene covered test tube racks, and an air tight plastic transport container.

Storage

Store test tubes in the refrigator at 4 °C or deep-frozen at -20 °C. Care should be taken with thawing: every time a container is opened, the risk of contamination will be enhanced. Sometimes tubes will leak after thawing. Discard these test tubes.

Sample preparation

Chemicals for the determination must be of Suprapur or comparable quality, for soaking p.a. quality may be used. Use water of bidistilled quality. Soak all laboratory ware in 1 + 10 diluted nitric acid. Dilute the sample with 1 + 1 or + 3 mol/L nitric acid, depending on the instrument used. Centrifuge subsequently at 2000 g. Transfer supernatant by pipetting and perform GFAAS determination. Evaluate against matrix adapted calibration solutions. Determine standards and samples in duplicate on two different days. Blank firings of water and diluted nitric acid must give zero absorbance. Calibration curves must consist of at least 4 points. Typical curves are dependent on the instrument used. Co-determine two (certified) reference samples for every 20 samples.

Instrumental conditions

Wavelength:	228.8 nm
Slit width:	0.5 - 0.7 nm
Lamp:	Hollow Cathode (current about 5 mA) or Electrodeless Discharge (power about 5 Watt)
Injected volume:	10 - 20 μL
Furnace:	pyrolytically coated tube with L'vov pyrolytically coated platform
Reading mode:	peak height or peak area
Sheathing gas:	nitrogen or argon
Background correction:	deuterium or Zeeman effect

Temperature program

	Temperature (°C)	Time (s)
Drying:	90 - 95	5 - 10
Pre-ashing:	120 - 150	15 - 30
Ashing:	300 - 400 maximum (critical)	40
Atomization:	1400 - 1500 zero sheathing gas flow	2.5 - 4
Cleaning:	2500 - 2600	3-4

Analytical parameters

Calibration curves linear to at least 40 pg.
Precision: Standard deviation = 0.1 μg/L
Bias (inaccuracy): on a level of 0 - 5 μg/L ± 0.3 μg/L; on a level of 5 - 10 μg/L ± 0.5 μg/L.
Detection limit: 0.04 - 0.08 μg/L

Determination of cadmium in urine

Considering the requirements of detection limit and contamination-free sample handling, determination of Cd-U with graphite furnace atomic absorption spectrometry (GFAAS)

is the method of choice. In an international intercomparison program for the determination of Cd-U, GFAAS was far the most used (Herber et al., 1990b). The detection limit of this most widely used method is about 0.02 μg/L (Herber, 1990b) and this enables the determination of the lowest concentrations reported of 0.05 μg/L (see also Table 3).

A standardized method for the determination of Cd-U will be mentioned here, including sample preparation. The method consists on dilution and acidification with nitric acid and subsequent direct determination (published more comprehensively by Herber et al. (1990b)).

Equipment and cleaning procedures

For urine use high density polyethene containers thoroughly cleaned with Suprapur quality nitric acid and bidistilled water 1 + 10. The container must be tested to deliver less than 10% extraneous amounts of cadmium. Test may be performed with a 1% v/v solution of nitric acid (e.g. Suprapur quality) in bidistilled water. Do not use glassware.

Urine collection

Voiding must take place in containers, wide enough to ensure contamination-free transfer of the urine. Take extreme care with working clothes like overalls. Pieces of dust may fall into the containers. Therefore ask workers to discard their working clothes before voiding the urine and to wash hands carefully during 2 min. If possible, collect 24 h urine. Problems arising here are the subjective collecting with respect to time and the enhanced risk on contamination due to the repeated opening and closing of the containers.

Storage

Sub-sample the urine as soon as possible (within 24 h) by using plastic pipettes. Do not touch the container or test tube upper walls with the pipettes. Shake vigorously, but without letting the urine come into contact with the upper wall container, before pipetting. Close the containers and test tubes as soon as possible after pipetting. Do not decant the urines. In case of determination by GFAAS, add as preservative a solution of HNO_3 until pH < 2. Store at 4 °C.

Evaluate Cd-U against aqueous or individual standard addition, dependent on instrument.Determine standards and samples in duplicate on two different days. Blank firings of water and diluted nitric acid must give zero absorbance. Calibration curves must consist of at least 4 points. Typical curves are dependent on the instrument used. Co-determine two (certified) reference samples for every 20 samples.

Instrumental conditions

See Cd-B. Peak measuring can be performed by peak height measurement.

Temperature program

	Temperature (°C)	Time (s)
Drying:	90 - 95	5 - 10
Pre-ashing:	120 - 130	15 - 30
Ashing:	400 - 450 maximum (critical)	10-40
Atomization:	1600 - 1800 zero sheathing gas flow	3-4
Cleaning:	2500 - 2600	

Analytical parameters

Calibration curves linear to at least 40 pg.
Precision: Standard deviation = 0.05 μg/L
Bias (inaccuracy): on a level of 0 - 5 μg/L ± 0.2 μg/L; on a level of 5 - 10 μg/L ± 0.4 μg/L
Detection limit: 0.02 - 0.04 μg/L

Determination of Cadmium in Hair

Cd-H offers no valuable information (see section on cadmium levels in body fluids). Therefore only a few remarks will be made about the determination. Collection of the hair is performed mostly by means of a tantalum pair of scissors from the occipital region. Storage may be done into polyethene tubes, acid washed. In the pre-analytical determination phase different washing procedures exist. A commonly used procedure is from the International Atomic Energy Agency (Ryabukhin, 1976) and consists of washing once with acetone, twice with bidistilled water and once with acetone. Subsequently the samples are dried in a stream of dust-free air. Homogenization may be performed in quartz tubes using a quartz rod under liquid nitrogen. Crushed aliquot must be allowed to reach equilibrium with air of known humidity for weighing. Determination may be performed with e.g. FAAS, DPPASV, GFAAS, neutron activation analysis, ICP, or solid sampling GFAAS. For FAAS and GFAAS destruction of the sample can be performed with 1 mol/L nitric acid (Herber, 1983). Subsequently evaluation of the samples against aqueous calibration curves can de performed.

Solid sampling GF-AAS can be performed directly after the washing procedure using solid reference samples as standard.

Determination of Cadmium in Tissues

Direct determination can be performed for special studies only; in this case a special neutron activation *in vivo* technique is used for determination of Cd in the kidneys or liver (Ellis et al., 1983, 1984).

Determination of placenta and autopsy material may be done with various techniques, e.g. FAAS, ICP, GFAAS, all after destruction of the sample. Destruction procedures are mentioned in the Chapter "Sample preparation".

A method applied for placenta tissue and compared with a solid sampling technique has been published by Herber et al. (1985).

Solid sampling GFAAS is able to determine the sample directly. The last technique is very suitable for both screening of the whole organ (e.g. the liver), or to locate "hot spots" e.g. in the kidney cortex. Examples are described by Pesch (1988) and Herber (1993b).

Determination of Cadmium in Faeces

Many techniques are available for the determination of Cd in faeces, e.g. FAAS, ICP, DPASV, GFAAS, etc. All methods require destruction of the sample preceding the determination.

QUALITY ASSURANCE

For internal quality control commercially available (certified) reference materials from e.g. BCR (Belgium, for Cd-B), Nycomed (Norway, for Cd-B and Cd-U), and NIST (U.S.A., for Cd-B and Cd-U) can be used. For tissue determinations many animal certified reference materials exist, e.g. from BCR and NIST. (see chapter on Reference Materials with a list of certfied reference materials)

External quality control for Cd-B and Cd-U is easier nowadays because (inter)national intercomparison programs are running frequently, e.g. the British program EQAS for Cd-B. Results from international quality control programs showed, that considerable problems with the determination still existed (Herber et al., 1990a, 1990b). A good approach to improve the quality of the determinations is the organization of workshops, courses, etc. in the laboratory where the determination has to be performed (Vahter, 1982; Herber, 1990c).

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Chapter 16

Chromium

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INTRODUCTION

The transition metal chromium (atomic mass 52) exists in a number of oxidation states, all of which are not of equal stability. The most common valences are 0, $+II$, $+III$ and $+VI$. Both Cr(III) and Cr(VI) are found in nature. From the values of the reduction potentials, together with thermodynamic energy considerations, it is evident that Cr(III) is the most stable form in solution. At cellular pH it is present as aquated $Cr_3(OH)_4^{5+}$, $Cr(OH)_2^+$ and $Cr(OH)^{2+}$ (Connett and Wetterhahn, 1983). It can also form complexes with many ligands. Cr(VI) occurs as CrO_4^{2-} and $HCrO_4^-$ (in biological systems the existence of $Cr_2O_7^{2-}$ is not expected to be significant) and as such behaves as a ligand. The distinction between the anionic and cationic forms of chromium is essential to the understanding of their divergent biological effects.

Chromium in biological material is probably always in oxidation state $+III$. The $+VI$ form is readily reduced to the trivalent form by contact with organic matter (De Flora and Wetterhahn, 1989). Oxidation of Cr(III) or Cr(0) to Cr(VI) in organic matter seems unlikely. Cr(VI) passes the cell membrane, while Cr(III) does not seem to have this property unless it is complexed to a low molecular weight compound. Once in the cell the Cr(VI) is reduced to Cr(III). The biological effects of Cr(VI) may thus be related to the reduction process, through the generation of reactive intermediates which ultimately bind to cellular constituents as Cr(III) and so damage their function in the cell (Connett and Wetterhahn, 1983; Norseth, 1981).

TOXICOLOGICAL AND ENVIRONMENTAL SIGNIFICANCE

Biological interest in chromium has been traditionally focused upon its toxic properties as an industrial hazard to man (Langård and Norseth, 1979; Norseth, 1986). It was about half a century ago that scientific and public concern arose over the dangers of increased incidence of bronchial cancer in workers in the chromate industry. Hazardous chromium exposure is also reported in metallurgical, refractory and chemical industries, which are

secondary users of chromium chemicals, such as in pigment and paint production, graphic art, galvanizing, plating, welding, machine drilling, cement production, timber and construction. An important consumer of Cr for many years has been the tanning industry. The major toxic effects of Cr are chronic ulcers, acute irritative dermatitis, allergic eczematous dermatitis, corrosive reaction in nasal septum, and local effects in the lung. Present evidence indicates that Cr(VI) plays an important carcinogenic role and that the reduction to Cr(III) assumes significance for the organ toxicity of the metal (Norseth, 1981; WHO, 1988; De Flora et al., 1990).

Chromium has now also become a common contaminant of the human environment. Possible sources of chromium exposure to the general public are emissions and waste dumps from the industry, causing local air and water pollution. Emissions from fossil-fuel burning also contribute to the atmospheric load. The presence of chromium in phosphates and in municipal sewage sludge used as fertilizers may be another important source of Cr in soil, in water and in some foods.

A completely different biological facet of the element chromium is that it may be essential for various forms of life, including man (Mertz et al., 1974). Very little can however be written with certainty about the exact biological role of Cr (Cornelis and Wallaeys, 1984). The problem is partly that many studies rely upon data without providing criteria satisfactory to ensure their reliability.

Excellent reference works on the biological and environmental aspects of chromium have been edited by Langård (1982), the World Health Organization (WHO, 1988) and Nriagu and Nieboer (1988).

ANALYTICAL CONSIDERATIONS

The life scientist interested in the concentration, distribution and speciation of chromium in biological systems is in need of reliable and precise analytical data. These can only be obtained by applying first of all a representative sampling and handling procedure, followed by the correct use of an analytical technique with suitable sensitivity. Finally, the bias of the whole method can be tested by analyzing biological standard reference materials, certified for chromium at comparable levels as the unknowns.

Although sampling and sample treatment are elaborately described in two previous chapters of this book, it may be interesting to give some more details that are specifically relevant for chromium. Problems related to the actual collection and the initial sample handling through to the analytical subsampling will be looked at more closely.

Exploration of μg/kg or ng/kg levels of Cr requires many precautions (Versieck and Cornelis, 1989). One of the primary concerns is that for clean room conditions. Belgian atmospheric aerosols contain 37 mg /kg Cr particulate matter, with a range of 8.8 - 150 mg/kg. Good analytical practice requires a fall-out below 1 μg/m^2.day in the working area. The use of a laminar flow hood, if possible a class 100 one, may offer an adequate solution at a modest price.

TABLE 1

Cr ADDITIONS TO HUMAN BLOOD COLLECTED WITH A DISPOSABLE STEEL NEEDLE
(Versieck et al., 1982)

Consecutive 20 mL fractions	Cr added μg/L
1	90
2	13
3	10
4	16
normal Cr serum	0.160

Evidently there is a fundamental need for impeccably clean recipients, made of plastic
or quartz. In general, direct contact with any metal or material containing chromium
should be excluded. This could be proven irrefutably by the following experiments (Ver-
sieck et al., 1982). Using neutron activated stainless steel needles for blood collecting and
Menghini biopsy needles and surgical blades for tissue sampling, several routine pro-
cedures were mimicked in vitro. In such conditions, the amount of radioactivity transferred
from the instruments to the samples reflects the contamination rate. Table 1 summarizes a
few data for Cr additions in blood samples, collected with disposable steel needles, as
compared to the intrinsic concentrations of Cr in serum. The additions are up to several
orders of magnitude. The same picture returns when liver biopsies are taken with
Menghini needles or with surgical blades, causing contaminations up to respectively 2000
and 3 times the intrinsic concentration of Cr in human liver (see Table 2).

TABLE 2

CR ADDITIONS TO FRESH TISSUE OF HUMAN LIVER BIOPSIES TAKEN BY ASPIRA-
TION WITH MENGHINI NEEDLE (M) AND WITH SURGICAL BLADE (WEDGE BIOPSIES)
(W) (Versieck et al., 1982)

Needle no.	Biopsy series	M mg/kg added	W mg/kg added
1	1	8.5	0.012
	2	1.9	0.010
2	1	11.0	0.020
	2	0.48	0.0028
intrinsic Cr content		0.0054	0.0054

TABLE 3

SOURCES OF Cr CONTAMINATION: LABORATORY WARE, ULTRAPURE ACIDS AND AMOUNTS LEACHED OUT BY HCl AND HNO_3 FROM PLASTICS (Kosta, 1982)

	Cr content	
Laboratory ware	$\mu g/kg$	
glass	10 000 - 100	
polyethylene	1 500 - 200	
teflon	100 - 10	
silica	100 - 10	
silica (suprasil)	3 - 0.2	
Ultrapure acids	$\mu g/L$	
HCl	0.01	
HF	0.1 - 0.01	
HNO_3	1 - 0.1	
$HClO_4$	1	
	Cr leached out after 1 week's contact (ng/cm^2)	
	6M HCl	9M HNO_3
Material		
polyethylene (HP)	10 - 1	1
polycarbonate	10 - 1	1
teflon	10 - 1	1

HP = high pressure

Cr is also a common constituent of laboratory ware and even of ultrapure acids. Data for glass, polyethylene, teflon, and silica can be found in Table 3, together with that for Cr impurities present in ultrapure acids. On extended storage these can possibly increase by a factor up to 10. Cr is leached out of the container walls, as has been proven by research done in this field by Moody and Lindstrom (1977). Table 3 summarizes some of their findings.

The above description mainly indicates contamination hazards as the major factor invalidating Cr analyses at the $\mu g/kg$ level. In addition liquid samples can also suffer from Cr losses through adsorption on the container wall. This depends upon the chemical composition of the sample (acidity, stability, concentration, chemical form, presence of ligands) and on the nature of the collection vial (material, surface).

Biological materials are liable to biochemical degradation processes as soon as they are collected. Freezing and diverse drying techniques are therefore common practice to keep the integrity of the specimens. A rule of thumb is to minimize the time lapse between the collection of the material and the subsampling. Liquid samples require either immedi-

ate analysis or else a fast processing in order to reach the frozen or inert dried state for storage as soon as possible. Trace element studies of e.g. urine have shown that Cr becomes greatly enriched in the sediment which gradually builds up after collection. It can, however, be brought into suspension again by vigorous shaking, but segregation promptly resumes (Cornelis et al., 1975).

The follow-up of the sample differs substantially depending on the analytical method. In some cases the sample can be analyzed as such or after simple dilution with water. This is the case for Cr determination in serum and urine with graphite furnace atomic absorption spectrometry. Freeze-drying of the biological specimen is sufficient for determining mg/kg amounts of Cr by e.g. instrumental neutron activation analysis. The detection of very low levels of Cr (μg or sub μg/kg, e.g. normal contents in serum, urine) requires a dry ashing step prior to irradiation. Cumulative evidence indicates that no perceptible losses of Cr occur if naturally labelled ^{51}Cr compounds are ashed up to 450°C (Versieck et al., 1979) or 500°C (Koirtyohann and Hopkins, 1976). Many researchers turn to wet digestion methods, either in an open system or in a closed pressure vessel. Low temperature ashing (LTA) has been increasingly used for biological samples. The method is, however, time consuming for large samples, but Cr can be recovered for 100 % (Williams, 1982). The main prerequisites for all procedures are ultraclean, high purity digestion vessels and ultrapure reagents in order to minimize the blank value.

The skill and experience of the analyst are decisive to guarantee the reliability and precision of the results. The following chapter deals in more depth with the different analytical methods currently used for Cr determination in biological materials. Once again, the importance of testing the bias of the Cr results by the analysis of suitable reference materials (certified for Cr present at a comparable level as the unknown) should be stressed. A list of reference materials can be found in the compilation by Cortes Toro et al. (1990), including the human serum reference material of Versieck et al. (1988). It may be said that the NIST brewers yeast SRM 1569 might be less recommendable because an important fraction of the Cr is bound to very inert components. As a consequence problems can arise when applying common digestion methods (De Goeij et al., 1978).

ANALYTICAL PROCEDURES

This section intends to give a survey of the more common analytical methods used in the determination of chromium in biological materials. The criteria which will be evaluated include precision, sensitivity, specificity and speed of analysis.

The yearly review articles in the Journal of Analytical Atomic Spectrometry provide an excellent follow up of the literature since 1985 (Brown et al., 1986, 1987, 1988, 1989; Crews et al., 1990, Branch et al., 1991, Taylor et al., 1992; Taylor et al., 1993). The following text only intends to give the state of the art in the field.

A survey of the literature showed that graphite furnace atomic absorption spectrometry (GFAAS) is by far the prevalent technique. Flame atomic absorption spectrometry (FAAS) has been superseded by GFAAS. The list of the other methods includes inductively

coupled plasma optical emission spectrometry (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), neutron activation analysis (NAA), chelate gas chromatography (GC), isotope dilution mass spectrometry (IDMS), X-ray methods such as particle induced X-ray emission (PIXE) and electrochemistry.

The most difficult Cr determinations are those at the μg and ng per kg levels, as present in body fluids. Such analyses are not only prone to many errors due to contamination, but also requires sensitivities below the detection limit of many methods. The μg/kg levels of e.g. soft tissues pose lesser problems than the mg/kg amounts of other specimens (food, hair, bone).

Atomic absorption spectrometry (AAS)

The application of AAS to the assessment of Cr in biological specimens has been extensively reviewed by Tsalev (1984) covering the literature from 1955 till 1981, and the state of the art on chromium determinations in environmental and biological samples by Rubio et al. (1992).

Flame atomic absorption spectrometry (FAAS)

The use of FAAS for Cr determinations in biological materials has become outdated. The sensitivity of most procedures was unsatisfactory and preconcentration steps were required in most cases. Therefore the early applications of FAAS will not be mentioned here. Only studies concerning Cr in specimens of exposed animals (e.g. faecal marker Cr_2O_3 (Gunčaga et al., 1974)) and Cr in certain foodstuffs or in leather can be reliably cited (see Table 4 and ref. Brown et al., 1986, 1987, 1988, 1989; Crews et al., 1990, Branch et al., 1991, Taylor et al., 1992; Taylor et al., 1993 for selected procedures). The use of a nitrous oxide-acetylene flame improves the selectivity of the Cr determination in comparison with the use of an air-acetylene flame, albeit at the expense of a somewhat

TABLE 4

EXAMPLE OF A FAAS PROCEDURE FOR Cr DETERMINATION IN BIOLOGICAL SAMPLES (extensive surveys can be read in Brown et al.,1986, 1987, 1988, 1989; Crews et al., 1990, Branch et al., 1991, Taylor et al., 1992; Taylor et al., 1993)

Sample	Brief procedure outline	Reference
leather meal	digestion of 2 g material (3 different acid digestion procedures and 1 dry ashing at 550°C were investigated); Perkin-Elmer 5000 FAAS (both N_2O-C_2H_2 and air-C_2H_2) were applied. Standardization versus Cr(VI) solutions. The air-C_2H_2 flame yields too low values and requires the standard addition method. Cr content, circa 3 %	Knecht (1983)

lower sensitivity. The detection limit at the 357.9 nm wavelength (spectral bandpass 1 nm) is described to be 0.02 mg/L in solution and 1 mg/kg dry matter (Hoenig and Borger, 1983). The Cr absorbance depends on the oxidation state, being higher for Cr(III) than Cr(VI) and careful matching of sample and standard solutions is necessary (Knecht, 1983).

Rubio et al. (1992) give a very interesting survey of the different kinds of interfering substances, such as cationic, anionic and acid matrices resulting from the digestion of the sample, which may influence the signal during FAAS with either air-acetylene, or nitrous oxide acetylene flame. In some cases ways to remove those interferences have been successfully explored.

Graphite furnace atomic absorption spectrometry (GFAAS)

At present Cr is regularly determined by GFAAS. Applications to urine and serum appear frequently in the literature. Hair, faeces, tissues, and various foodstuffs are also analyzed for their Cr content (see Table 5 for more detail).

Veillon et al. (1980) studied the retention of Cr by graphite furnace tubes. Using endogenously ^{51}Cr labelled rat urine as well as ^{51}Cr solutions, a considerable amount of Cr was found to be irreversibly retained in the graphite furnace tubes upon atomization. Pyrolytically coated tubes retained less Cr than uncoated tubes. The amount of Cr retained depended on the matrix and on the atomization temperature. The authors therefore concluded that major errors are likely to occur if the method of standard additions is not used. No Cr loss by volatilization was observed for thermal pretreatment up to 1300°C. Similarly, Arpadjan and Krivan (1988) studied the behaviour of chromium by means of ^{51}Cr radiotracer in the graphite furnace during the performance of flameless absorption spectrometry. They investigated various biological matrices, such as human serum, milk, and cow milk, and observed no differences between uncoated and pyrolytically coated tubes. Significant stabilization effects could be achieved in the pre-atomization step by using tungsten-coated tubes and/or matrix modifiers of which Na_2WO_4, Na_2MoO_4 and H_2O_2 proved to be well suited. The results indicated that in optimized experimental conditions no strong retention on the surface of the graphite tube by carbide formation takes place.

In case of the ng per L Cr amounts, as present in e.g. blood plasma, serum and urine, the main problems are avoiding contamination (mentioned earlier in this chapter) and correcting accurately for background absorption. As a matter of fact, the background correction capability of the available atomic absorption spectrometer is decisive for the bias of the analytical result. The Cr absorption is measured at the 357.9 nm line. At this wavelength region the intensity of a deuterium lamp is very low in comparison with that of the hollow cathode lamp (HCL). To balance the intensities of the two sources, the HCL current has to be drastically reduced. This decreases the sensitivity and moreover, the background emission of the incandescent graphite tube may give rise to additional emission noise (Davidson and Secrest, 1972). Therefore the use of a modern graphite furnace atomic absorption spectrometer with enhanced background correction capabilities is in-

346   R. Cornelis

TABLE 5

SELECTED GFAAS PROCEDURES FOR Cr DETERMINATIONS IN BIOLOGICAL
SAMPLES (extensive surveys can be read in Brown et al.,1986, 1987, 1988, 1989; Crews
et al., 1990, Branch et al., 1991, Taylor et al., 1992; Taylor et al., 1993)

Sample	Brief procedure outline	Reference
beer, beer ingredients, urine	beer samples diluted at least 4-fold with H_2O; beer ingredients extracted with 0.1 mol/L NH_4OH; urine assayed directly; Perkin-Elmer 5000, HGA 500; pyrolytic coated tubes, standard addition calibration. Furnace conditions for beer: drying 100°C, ramp 10 s, hold 20 s; internal argon drying flow, 300 mL/min; second drying, 130°C, ramp 15 s; hold 10 s; internal argon flow, 300 mL/min; ash 1100°C, hold 30 s; atomize, 2700°C, ramp 0 s, hold 4 s, internal gas flow, 50 mL/min; clean out, 2700°C; ramp 1 s, hold 4 s; internal gas flow 300 mL/min. (for urine: see bottom of table, urine publication by Veillon et al., 1982). Cr concentration of the selected beers ranged from 0.48 to 56 μg/L.	Anderson and Bryden (1983)
hair	hair dry-ashed overnight at 500°C in Vitreosil quartz crucibles; ash treated with a little H_2SO_4 and 30 % H_2O_2; ash dissolved in 1 mL 1 mol/L HCl; Cr determined against Cr standards; Perkin-Elmer 5000 with background corrector (tungsten iodide lamp), furnace HGA 500. Experimental conditions, see publications listed at human milk, serum, yeast by same first author; concentration of Cr in hair, about 0.1 mg/kg	Kumpulainen et al. (1982)
human milk, serum, urine	breast milk samples are diluted 1 + 1 and serum 1 + 2 with water; urine without preliminary dilution. The method of additions is used for all samples analyzed. For each determination 10 μL of 1 mol/L HCl is added to 1.0 mL of sample, followed by 10 μL additions of standard solutions of 25, 50 and 75 μg/L Cr to 3 successive cups with 1.0 mL sample. Perkin-Elmer 5000 AAS, tungsten-iodide lamp background correction, HGA 500 furnace, wavelength: 357.9 nm; slit 0.7 nm; mode: peak height; integration time 4 s. Furnace program, 1[rst] dry 120°C, hold 40 s, ramp 2 s; 2nd dry 500°C, hold 10 s, ramp 5 s; ash 1100°C, hold 60 s, ramp 5 s; atomize 2700°C, hold 3 s, clean 2800°C, hold 2 s. Detection limit: 0.1 g Cr/L Cr for human milk; 0.05 μg/L Cr for serum and urine. Typical concentrations: 0.49 μg/L for breast milk, 0.12 μg/L for serum and 0.11 μg/L for urine.	Kumpulainen et al. (1983)

TABLE 5 (continued)

Sample	Brief procedure outline	Reference
plant tissue	≤1 g sample is digested in HNO_3, $HClO_4$ + H_2SO_4; Cr is coprecipitated on $Fe(OH)_3$; Fe extracted with 4-methyl-2-pentanone; silica dissolved with HF and Cr measured with GFAAS versus standard curve; pyrolysed tubes are used. Perkin-Elmer 603 with HGA 2100 furnace, drying 110°C for 20 s, charring 1000°C for 10 s; atomizing 2700°C for 9 s, argon flow normal, flow rate 40. No background correction applied. Cr values determined are of the order of several mg/kg Cr dry material.	Cary and Rutzke (1983)
serum	1-2 mL serum are weighed into silanized (with di-chloro-dimethylsilane) quartz tubes in the presence of 1.86 mg $Mg(NO_3)_2.6H_2O$ per mL serum, followed by lyophilization, overnight dry ashing at 480°C; dissolution of the ash in 0.1 mol/L HCl, detection of Cr by GFAAS. Perkin-Elmer 5000, with tungsten-halogen lamp for background correction; HGA-500 furnace; pyrolytically coated furnace tubes: lamp current 25 mA. Furnace program: first dry 100°C, hold 20 s, ramp 15 s; second dry 140°C, hold 20 s, ramp 10 s, char 1350°C, hold 30 s, ramp 15 s, atomize 2700°C, hold 4 s, ramp 0 s, clean 2700°C, hold 4 s, ramp 1 s. Internal flow 50 mL/min. Method of standard additions is not required. Calibration curves prepared with a bovine serum pool to which known amounts of Cr have been added. Blank value: 0.02 μg/L, detection limit about 0.03 μg/L; typical Cr value with standard deviation: 0.27 ± 0.02 μg/L.	Veillon et al. (1984)
tea, mussel, oyster tissue	direct determination of Cr in the samples by Zeeman AAS with graphite miniature cup, peak height method, conditions: preashing at 220°C for 60 s; ashing at 550°C for 120 s of powder samples (plants) or 1050°C for 120 s for oyster tissue and mussel, atomization at 2500°C for 10 s. Calibration for Cr: range 0 to 4.5 mg/kg were established with NBS SRM's. The values investigated for tea varied between 0.48 and 0.74 mg/kg. Sensitivity: 0.007 ng.	Itoh et al. (1984)
urine	50 mL urine digested with 15 mL HNO_3, 3 mL H_2SO_4, oxidation of all Cr to Cr(VI) with 0.002 mol/L $KMnO_4$, adjustment pH = 4 with NH_4OH; extraction of Cr in 2 % (w/v) ammonium pyrrolidine-dithiocarbamate in water into 5 mL methyl isobutyl ketone. Calibration curve versus a synthetic urine, similarly treated, spiked with Cr, no background correction applied; pyrolytically coated tubes. Shimadzu AA-640-13 AAS with GFA-3 furnace.	Ping et al. (1983)

TABLE 5 (continued)

Sample	Brief procedure outline	Reference
urine	Program setting, dry 150°C, hold 30 s; 800°C, hold 30 s; atomize 2500°C, hold 4 s. The method was compared to the direct determination of Cr in urine (requiring a twice longer ashing). The results do agree. Detection limit in both cases appeared to be 0.05 μg/L. The urinary concentrations reported are about 1 μg/L. reliable results require the method of additions, 1 mL urine is spiked with 10 μL solutions of 0, 20, 50 and 100 μg/L Cr in 1 mol/L HCl. Perkin Elmer 5000, graphite furnace HGA 500, pyrolytically coated furnace tubes, argon, tungsten chloride lamp for background correction. Furnace program dry 1 at 100°C for 20 s, ramp 15 s; dry 2 at 130°C for 20 s, ramp 10 s; char at 1200°C for 6 s, ramp 15 s; atomize at 2700°C for 4 s; clean out at 2700°C for 4 s, ramp 1 s; internal flow 50 mL/min. Detection limit 0.03 μg/L. Concentrations circa 1 μg/L.	Veillon et al. (1982)

dicated (Veillon et al., 1982). The Zeeman background correction is to be prefferred at this point (Fernandez et al., 1980). Background absorption can be reduced still further by adding as a modifier/analyte 1 or 2 % v/v HNO_3 or HCl modifier (Routh, 1980).

It is essential to have some means of multi-stage temperature programming including very fast "maximum power" heating during the atomization stage and a good control of the flow rate of the purge gas (Chao, 1980).

Many problems may occur when performing GFAAS of Cr in biological samples (Tsalev, 1984). Matrix-residue built-up is quite common together with memory effects. Brushing-out of the matrix residues may be necessary after every few samples e.g. in case of serum. Significant tube to tube variations of the absorption signal can be expected. Relatively long temperature programs (5 to 6 min) are required. The analyte signal may be enhanced in the biological matrix (e.g. serum, urine) versus aqueous standards. It is impossible to describe a universally applicable procedure for Cr determinations in biological materials, due to the large variability in the intrinsic quality of the equipment and to the preponderant role played by the matrix. As stated previously the latter problem requires the standard addition method.

Generally speaking, modern GFAAS apparatus is sufficiently sensitive for the determination of Cr in biological specimens at the 0.1 - 0.2 μg/L level so that preconcentration steps are not required. Table 5 lists a selection of GFAAS procedures for Cr in biological samples, either directly or after simple dilution, or including an adequate digestion. This compilation is limited in its size. The procedures cited can, however, be

easily transposed to any other biological material. When doing so, adjustment of the digestion procedure and/or the furnace conditions will be needed in most cases.

A ~~recent~~ development in GFAAS is the application of absolute - actually referred to as standardless - stabilized platform furnace (STPF) AAS (Slavin et al., 1984) as described in another chapter of this book. Slavin et al. (1983) review their experience and that of other researchers with the STPF technique for 34 analytes, including Cr in biological specimens. They describe how the addition of $Mg(NO_3)_2$ increases the temperature at which Cr is volatilized to 1700°C. Apparently the presence of Mg oxide, formed at high temperature from $Mg(NO_3)_2$, sequesters the Cr and prevents its volatilization until the Mg oxide itself has volatilized. Together with Zeeman background correction a detection limit of 1 pg or 0.05 μg/L in a 20 μL sample can be obtained in any matrix that can be pipetted on the platform. With STPF conditions and $Mg(NO_3)_2$ there was no difference in the Cr signal between standards made up of Cr(III) and Cr(VI), and there was no interference to be noticed from Ca when integrated absorbance signals were used, which forms part of the STPF methodology. Slavin et al. (1983) determined Cr in 1 + 1 diluted urine with a detection limit of 0.1 μg/L in 20 μL aliquots. To improve the detection limit, five aliquots were fired, with pyrolysis between each aliquot so that 0.02 μg/L becomes feasible.

Rubio et al. (1992) also report on the various possible interferences of cations, anions and acids during GFAAS measurement and feature the qualities of the various types of tubes.

Inductively coupled plasma-optical emission spectrometry (ICP-OES)

The recent literature shows a rather modest use of ICP-OES for Cr determination in biological specimens, with a view to make it part of a multi-element scheme. The detection limit for the Cr 267.716 nm analytical line is described as being 6.0 μg/L, exhibiting a possible spectral interference by V (Schramel, 1983). Broekaert stipulates 20 μg/L Cr as a detection limit at the 283.6 nm line (Broekaert, 1987). Such detection limits are, however, insufficient for biomedical applications, as body fluids (e.g. serum, urine) contain less than μg/L levels. Consequently, preconcentration steps are necessary to attain those low levels. When determining Cr in biological samples, an adequate software for quantitative spectral analysis (including background correction) is indispensable as this greatly determines the reliability of the analytical results. Table 6 gives a few examples of Cr analysis procedures by ICP-OES.

Inductively coupled plasma - mass spectrometry (ICP-MS)

Interestingly enough, ICP-MS offers a detection limit for Cr of 0.11 μg/L on the most abundant nuclide ^{52}Cr ($\Theta = 83.79\%$) (Vanhoe, 1992). The occurrence of major spectral and matrix interferences (Hywel Evans and Giglio, 1993) nearly excludes the use of ICP-MS for the low Cr determinations as present in biological samples. Due to the presence of Cl and the formation of the polyatomic ClO^+ and $ClOH^+$ compounds the Cr determination is in serious jeopardy. The spectral overlap of $^{35}Cl^{17}O$ and $^{35}Cl^{16}O^1H$

TABLE 6

SOME ICP-OES PROCEDURES FOR Cr DETERMINATIONS IN BIOLOGICAL SAMPLES
(extensive surveys can be read in Brown et al.,1986, 1987, 1988, 1989; Crews et al., 1990,
Branch et al., 1991, Taylor et al., 1992; Taylor et al., 1993)

Sample	Brief procedure outline	Reference
marker Cr in faeces	1 g dry faecal material are digested in $HNO_3 + HClO_4$ Labtest Plasmascan 700 ICP-emission spectrometer; 358 nm; calibration standard solutions; detection limit 0.013 mg/L, limit of quantitation 1.4 mg/kg; concentration found: circa 2 g/kg.	Roofayel and Lyons (1984)
urine, dextrose	speciation of Cr through binding at pH 5 of Cr(VI) on polydithiocarbamate and of Cr(III) on polyacryl-amidoxime. Resins digested in HNO_3 and measured with ICP-OE spectrometer; wavelength 283.5 nm; standard addition method, using 500 mL urine sample or a 50 mL dextrose; the detection limit is 0.028 μg/L and 0.28 μg/L respectively; limit of quantitation 0.140 μg/L and 1.4 μg/L, respectively. Concentration in urine < 1 μg/L; in dextrose 10 μg/L. In urine Cr is exclusively present as Cr(III).	Mianzhi and Barnes (1983

hinders the determination of ^{52}Cr, and $^{37}Cl^{16}O^+$ that of ^{53}Cr ($\Theta = 9.5\%$). The use of Ar as carrier gas and plasma source in the presence of large amounts of C causes additional problems, as the formation of $^{40}Ar^{12}C$ interferes with the ^{52}Cr determination and that of $^{40}Ar^{13}C$ with the ^{53}Cr determination. Some researchers tried to circumvent this difficulty by using an alternative plasma source. Besides the Ar, C, Cl and O interferences already cited, there is also the possibility of forming polyatomic compounds with S through $^{34}S^{16}O$ and $^{32}S^{18}O$, interfering with ^{50}Cr. Further on the nuclide ^{54}Cr is subject to interference by ^{54}Fe and by $^{37}Cl^{17}O$ and $^{37}Cl^{16}O^1H$. With the advent of high resolution ICP-MS, these interferences are not expected to arise.

Neutron activation analysis (NAA)

NAA of Cr in biological materials is an excellent analytical method which offers a low bias and suitable sensitivity. It is, however, more time consuming than GFAAS and requires an elaborate infrastructure. Therefore its application is never oriented towards routine work, but focuses on research purposes. NAA is also often used as a reference method. The nuclear reaction involved is $^{50}Cr(n,\gamma)^{51}Cr$ ($t_{1/2} = 27.7$ d, γ 320 keV, cross section σ 15.8 barn; isotopic abundance of ^{50}Cr: 4.4 %).

Generally, NAA *offers the advantage that sample treatment prior to irradiation can be* minimized. Subsampling and packing in polythene vials or quartz ampoules are the sole manipulations. Exogenous contaminations due to reagents are not to be feared after irradiation, an important asset if compared to most other analytical techniques.

Very low concentrations of Cr (μg and ng per kg levels) require, however, long irradiation periods at a high neutron flux, followed by a radiochemical separation. Due to radiation damage induced high pressure built-up, the sealed ampoule can explode. This is avoided by ashing the sera in the quartz vial at a temperature of 450°C, prior to irradiation (Versieck et al., 1978).

The reliability of the determination at the ng/kg levels of Cr may be jeopardized by a blank value caused by the irradiation vial (Cornelis et al., 1982). Even using the highest purity quartz vials available, and however perfectly the surface of the vials has been cleaned, a small number of atoms of the Cr impurity present in the irradiation vial become radioactive during neutron bombardment and are liable to be ejected from their original sites. As a result of this recoil effect, ^{51}Cr ends up in the sample, moreover as the ash has to be washed out of the container with strong acids after irradiation. Some typical Cr blank values from quartz containers are described in the literature. After 14 d irradiation at 10^{14} n/cm^2.s (BR-II reactor Mol, Belgium) "Spectrosil" quartz ampoules opened by snap cutting yielded a mean of 0.0478 ng Cr (range 0.0262-0.074 ng) and Heraeus "Suprasil" quartz about 0.020 ng Cr. In case of Cr determinations in a 1 mL human serum sample, normally containing 0.16 μg/L Cr, such blank values become quite substantial.

It may be interesting to draw attention to a possible nuclear interference for Cr determinations when large amounts of Fe are present in the biological sample. In principle NAA with a nuclear reactor assumes that ^{51}Cr can only be formed through (n,γ) reaction on [50]Cr. The reactor neutron spectrum also contains fast neutrons, which can give rise to threshold reactions of the type $^{54}\text{Fe}(n,\alpha)^{51}\text{Cr}$ (cross section $\sigma_f = 0.74$ mb). The unreliablility of Cr determinations in the presence of several orders of magnitude higher Fe concentrations can be illustrated in the case of red blood cells. These contain about 1.02 g/kg Fe, which in a thermal:fast flux ratio of 6.4 produces an apparent 11 μg/kg Cr, one order of magnitude higher than the actual Cr content.

The sensitivity of NAA for Cr determinations depends largely on the irradiation facilities (neutron flux and duration), the efficiency and the resolution of the detector for the subsequent γ-spectrometry *as well as on the presence of other long-lived radioisotopes.* The sensitivity and the accuracy can always be greatly improved by a radiochemical separation of the Cr as then the low ^{51}Cr activity, 320 keV photopeak, will be freed from the background Compton continuum of ^{59}Fe, ^{60}Co, [65]Zn, ^{86}Rb, [134]Cs, and from the [32]P Bremsstrahlung. An irradiation of 14 d at 10^{14} n/cm^2.s, followed by a radiochemical separation and Ge(Li) γ-spectrometry, can yield sensitivities as low as 0.01 to 0.005 ng Cr. The choice of the radiochemistry (extraction, distillation, ion exchange) is rather free and *usually occurs in the presence of Cr carrier. Biological samples containing mg/kg down to* 10 μg/kg Cr amounts are generally processed in a purely instrumental way.

Table 7 lists a very limited selection of NAA procedures for Cr in various biological matrices, either by instrumental NAA (INAA) or after radiochemical separations. In both

TABLE 7

SELECTION OF NAA PROCEDURES FOR Cr DETERMINATIONS IN BIOLOGICAL
SAMPLES

Sample	Brief procedure outline	Reference
brain	INAA: 10 to 15 mg freeze-dried tissue is irradiated in suprasil quartz vials for 40 h at a flux of $3.5.10^{13}$ $n/cm^2.s$; cooling period 3 to 4 weeks; γ-spectrometry on Ge(Li) detector. Mean concentration: $127 \pm 13 \mu g/kg$.	Ehmann et al. (1982)
food composite	INAA: aliquots of freeze-dried 1 day food composite are irradiated as pellets for 72 h at 6.10^{13} $n/cm^2.s$ cooling period 6-8 weeks; Ge(Li) γ-spectrometry; median daily Cr intake estimated at $47 \mu g$.	Gibson and Scythes (1984)
hair	INAA: 20-180 mg washed hair irradiated for 7 h at 1-2.5 10^{12} $n/cm^2.s$; cooling period: 30 d; Ge(Li) γ-spectrometry; mean Cr value of circa 0.8 mg/kg.	Tomza et al. (1983)
lung	INAA: 300 mg dry material is irradiated for 31 h at 2.10^{12} $n/cm^2.s$; cooling period 20 d; Ge(Li) γ-spectrometry. Range of Cr values investigated: 16-400 $\mu g/kg$ wet lungtissue.	Vanoeteren et al. (1982)
serum	Radiochemical NAA: 100 mg lyophilized serum are ashed in a quartz ampoule during 24 h at 100, 200, 350 and 450°C successively; sealed ampoules irradiated for 12 d at 10^{14} $n/cm^2.s$; cooling period: 30 d; outside contaminations of ampoule removed with HF (50 %) and HNO_3 (14 mol/L); content dissolved in 3 mL equivolume mixture of $HClO_4$ (70 %) and HNO_3 (14 mol/L); Cr distilled in the presence of 5 mg Cr carrier in 10 mL $HClO_4$ as CrO_2Cl_2 at 200°C while dry HCl gas is introduced. Distillation repeated twice after addition of fresh 5 mg Cr carrier. Ge(Li) γ-spectrometry. Mean Cr value: 0.16 $\mu g/L$ serum.	Versieck et al. (1978)
serum	Radiochemical NAA: 150 mg lyophilized serum are ashed, irradiated and dissolved as described above; the solution is eluted consecutively over acid aluminium oxide (AAO), hydrated antimony pentoxide (HAP) and hydrated manganese dioxide (HMD); Cr is retained on the HMD column and counted on the packing material; 2 methods for calibration were applied: the k_0-method and the relative method versus a Cr-standard.	Lin Xilei et al., 1988

TABLE 7 (continued)

Sample	Brief procedure outline	Reference
biological material: whole blood, liver, milk powder	Radiochemical NAA: irradiation for 8 - 16 h at 2.7. 10^{13} n/cm^2.s, digestion along with 1 mg of Cr-carrier (as $K_2Cr_2O_7$), 20 mL HNO_3, 3 mL H_2SO_4 and a few drops of HF in a teflon TFE dissolution vessel; addition of 10 mL HNO_3, reheated and again 10 mL HNO_3 and 5 mL H_2ClO_4 till fuming; after cooling 5 mL H_2O is added as well as 1 mL of a solution containing 10 g/L of $KMnO_4$ in order to oxidize any remaining Cr to Cr(VI); 20 mL of 1.5 mol/L HCl is added and Cr(VI) is separated by liquid-liquid extraction of Cr(VI) into a solution of tribenzylamine/chloroform and back extracted into 2 mol/L NaOH.	Greenberg and Zeisler (1988)

circumstances the Cr determinations are more often than not part of a multi-element scheme.

Isotope dilution mass spectrometry (IDMS)

IDMS is an excellent reference method for the determination of Cr in biological specimens. The sample is spiked with ^{50}Cr and Cr is separated before detection. The method can be successfully applied for the determination of very low Cr values. Table 8 gives an example.

TABLE 8

AN IDMS PROCEDURE FOR Cr DETERMINATION IN BIOLOGICAL SAMPLES

Sample	Brief procedure outline	Reference
serum	15 mL serum are weighed in a silanized quartz tube in the presence of ^{50}Cr spike and of $Mg(NO_3)_2$, lyophilized and ashed at a final temperature of 480°C. The ash is dissolved in NH_4Ac buffer and Cr chelated with trifluoroacetylacetone. Measurement of the ^{50}Cr to ^{52}Cr isotope ratio by combined gas chromatography and dual ion monitoring. Serum concentration: 0.27 μg/L.	Veillon et al. (1984)

X-ray spectrometry

This multi-element method includes Cr. The sensitivity is however influenced by the matrix components and the subsequent background emitted by major components. Particle induced X-ray emission (PIXE) analysis with the aid of a Si(Li) detector is occasionally used for Cr determinations in biological specimens. The sensitivity is too low to measure the normal Cr content of biological fluids (i.e. less than μg/L levels), but the method yields reliable data for the 1 mg/kg amounts (Maenhaut et al., 1984).

Electrochemical methods

Differential pulse polarography is used as part of a multielement procedure for the determination of Cr in foods. The sensitivity is described as 0.24 mg/kg Cr (Holak, 1983). Trace amounts of Cr have also been determined by using the catalytic current at a dropping Hg electrode (Sarawathi et al., 1990). The determination of trace amounts of Cr(VI) and/or Cr(III) or total Cr by electrochemical methods are described by Pratt and Koch (1986), Boussemart and Van den Berg (1991), and Elleouet et al. (1992), to cite just these few.

Chromatographical methods

Gas-liquid chromatography is mentioned in the literature for the determination of Cr in various biological materials, using β-diketones as chelating agent. The method includes the possibility of distinguishing among different oxidation states. Increased selectivity over gas-liquid chromatography can be gained by high performance liquid chromatography (HPLC) (Willett and Knight, 1982). The latter method combined with mass spectrometry offers an on-column detection limit of 1 ng.

Reversed phase HPLC was used for a mixture of trace elements, including Cr(III), chelated with 2-(2-thienylazo)-5-diethylaminophenol on a C_{18}-bonded stationary phase (Liu et al., 1992). The detection is reported to be 2 μg/L Cr(III). The method was applied for the analysis of Cr in tea leaves and rice.

SPECIATION OF CHROMIUM

This subject is reviewed more in detail in the chapter on speciation by P.E. Gardiner and H.T. Delves.

Speciation of Cr in biomaterials means differentiation between the various species of the element. In fact this can be understood in two ways. Firstly speciation can be studied by separating the proteins and analyzing the amount of Cr in them. Secondly it can consist of a search for the oxidation state in which Cr is transformed or bound to macromolecular compounds. These studies are difficult because of the variety of chemical compounds in biological materials which interact with Cr.

Several speciation procedures for Cr(III) and Cr(VI) can be found in the literature. They are based on the distinction between cationic Cr(III) and anionic Cr(VI) forms.

Mianzhi and Barnes (1983) describe a method that starts with two distinct samples of the biological fluid. One is treated with polydithiocarbamate (PDTC) resin at pH 5, which binds the Cr(VI), the second is treated with polyacrylamidoxime (PAAO) resin, also at pH 5, to bind the Cr(III). The resins are subsequently digested in HNO_3 and the Cr content is measured by ICP-OES.

Minoia et al. (1983) used a liquid anion exchanger Amberlite LA 1 or LA 2 in isobutyl methyl ketone and 6 mol/L HCl mixture, which was added to the aqueous biological specimen (in casu urine). After extraction of the anions, centrifugation and separation of both phases, all Cr(VI) was bound to the organic phase, whereas Cr(III) was present in the aqueous phase.

Such a speciation of the oxidation state is, however, only valid for pure ionic Cr compounds. It is ideal for non protein bound Cr. Whenever other ligands are present, this method supposes a stronger binding with the resins than with the ligand.

When performing separation of the species, the concentration of the Cr is diluted, more often than not below the detection limit of the measurement technique. In this case a preconcentration step is mandatory, preferably on-line. Such a system has been developed for the determination of Cr(III) and total Cr by Sperling et al. (1992).

The previously described cationic and anionic tests are jeopardized when the Cr is build into a compound as Cr(III), while this carries a negative charge. It behaves like an anion and may lead to false conclusions about the valence state of Cr. A more valid use of the above mentioned tests for Cr(III) and Cr(VI) species requires a preliminary clearance of all proteins from the solution (e.g. through ultrafiltration or ultra-centrifugation).

Speciation of Cr as part of biomolecules consists in applying well known biochemical procedures, such as gel permeation chromatography, anion - cation chromatography, affinity chromatography, capillary electrophoresis, ultrafiltration, to be followed on the one hand by the determination of the exact amount of a specific protein and on the other hand by the quantitative determination of the chromium present in the fractions (Cornelis, 1990). This is not an easy task. All the commercially available buffers are unfortunately loaded with Cr. The separation medium (e.g. the gel) may be another possible source of error. The contaminating Cr atoms are liable to form fortuitous bindings with the proteins which possess many free ligands.

These difficulties due to contamination can be partly circumvented by use of in vivo labelled ^{51}Cr compounds (Cornelis, 1992; Cornelis et al., 1992). Nevertheless a lot of care should be taken to add only negligible amounts of exogenous Cr carrier in order not to disturb certain equilibria. As a matter of fact, very interesting knowledge on the kinetics of Cr transport in biological systems has been obtained with in vivo as well as in vitro experiments with ^{51}Cr labelled compounds. Lim et al. (1983) were able to shed new light on the metabolism and nutritional requirements of Cr(III) in the human body. Numerous animal experiments with [51]Cr(VI) and [51]Cr(III) are on the verge of unravelling the cellular metabolism of Cr (e.g. Tsapakos and Wetterhahn, 1983; Manzo et al., 1983, Borguet et al., 1994b, to cite just these 3 publications). In case of serum it was shown that Cr is

mainly bound to transferrin - and to a lesser degree - to albumin (Wallaeys et al., 1987). Borguet et al. (1991; 1994a) developed a separation method for the speciation of Cr in plasma of patients on continuous ambulatory peritoneal dialysis consisting of FPLC (Fast protein liquid chromatography) cation- and anion exchange with a complete resolution of the Cr-binding proteins and a quantitative recovery of the proteins as well as the Cr.

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Copper

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INTRODUCTION

Copper, atomic number 29 and atomic mass 63 is the 26th most abundant element in the earth's crust. It has been known for about 10,000 years and occurs in pure form as a "semiprecious metal" or in compounds as Cu(I) or Cu(II). Copper metal has a mass density of 8,930 kg/m^3, a melting point of 1083 °C and a boiling point of about 2590 °C. Natural copper consists of 69.1% ^{63}Cu and 30.9% ^{65}Cu. It can be easily manufactured from sulphidic (e.g. CuS, covellite; Cu$_2$S, chalcolite; CuFeS$_2$, chalcoprite; Cu$_3$FeS$_3$, bornite) or oxidic (e.g. Cu$_2$CO$_3$(OH)$_2$, malachite; CuSiO$_3$x2H$_2$O, chrysocolla; CuSO$_4$x5H$_2$O) ores. Copper ores contain usually other metals such as Zn, Fe, As, Cd etc. Copper is after silver the best common conductor of heat and electricity. Copper metal is further used in various applications, e.g. for water piping, stills, roofing material, kitchenware, and for chemical and pharmaceutical equipment. There are several alloys of copper such as brass (with zinc), bronze (with tin), monel metal (with nickel) etc. Copper compounds are used in various applications including many technical. Copper salts are used as fungicides but also as part of animal feed (swine and poultry) to promote growth (Scheinberg, 1991; Triebig and Schaller, 1984).

ESSENTIALITY AND TOXICITY

Copper is one of the several heavy metals that have essential as well as toxic properties. Copper is toxic to many bacteria and viruses but for plants copper toxicity is virtually

unknown. Copper is an essential element for human beings. It is an essential part of several enzymes including ferroxidases, cytochrome oxidase, superoxide dismutase, and amine oxidases. Excess copper ingested in the diet of man and animals usually is deactivated or eliminated by various biochemical mechanisms. Thus under normal conditions (i.e. intake is not too excessive) no toxic actions occur. "Copper is probably only toxic to man and animals when one of these mechanisms is defective, either because of genetic or acquired causes" (Scheinberg, 1991). Copper is also to some extent essential to plants and animals; the latter may develop deficiency symptoms if feed does not contain a sufficient amount of copper. Usually, with rare exceptions, copper deficiency in man does not occur.

Accidental ingestion of copper compounds may lead to acute intoxication with gastrointestinal disturbances. Very high doses might be letal. Chronic copper exposure in industry, e.g. inhalation of copper fumes, may lead to various health effects like respiratory symptoms, gastrointestinal disturbances, nervous dysfunctions, dermatological and hematological changes etc. (Triebig and Schaller, 1984; Aaseth and Norseth, 1986; Scheinberg, 1991).

In Wilson's disease, an autosomal recessive inborn error of human metabolism, serum ceruloplasmin levels are markedly reduced, and excess copper deposits occur in parenchymal tissues, mainly the liver. Ceruloplasmin levels in healthy persons range from 200-500 mg/L, whereas patients with Wilson's disease have less than 200 mg/L (Scheinberg, 1983). In these patients the Cu-S concentrations might be only one-half of normal, whereas liver concentrations are five fold and urine levels are ten fold normal (Fell et al., 1968). The disease is progressive and fatal, if not treated by a de-coppering regime. Scheinberg (1983) proposed a screening in pre-employment health examination to exclude persons from occupational exposure to copper, which show subnormal ceruloplasmin levels in serum. In Menkes' syndrome, abnormally low copper levels are found in liver and most extrahepatic tissues. In such patients a large percentage of the small amount of copper absorbed accumulates in the kidney, presumably after reabsorption (Aaseth and Norseth, 1986).

COPPER LEVELS IN BODY FLUIDS AND TISSUE

The normal mean plasma or serum level of copper lies around 1.1 mg/L (Triebig and Schaller, 1984; Versieck and Cornelis, 1989). The mean urinary levels reported by Versieck (1985) range from 15 to 36 μg/24 h. This is in excellent agreement with the estimate of Triebig and Schaller (1984). The latter reported a mean urinary daily excretion of around 20 μg and an upper limit of copper concentration in urine of 50 μg/L. These levels might be influenced by physiological or pathological conditions. Recently, Minoia et al. (1990) recorded as mean values for copper in blood 1.2 mg/L (n = 475; range 0.8 - 1.6 mg/L), serum 0.985 mg/L (n = 901; range 0.6 - 1.37 mg/L) and urine 23 μg/L (n = 507; range 4.2 - 50 μg/L). According to Lauwerys and Hoet (1993) the data concerning

relationship between occupational exposure, internal dose and effect are "at present too scarce to suggest reliable biological limit values".

Selected reference values for copper in various human tissue of healthy subjects range, according to Versieck (1985) for liver from 2.6 to approx. 14 mg/kg, for kidney cortex from 1.4 to approx. 3.9 mg/kg, and for muscle tissue from 0.6 to 1.0 mg/kg. All data were given for wet weight.

ANALYTICAL CONSIDERATIONS

Low bias, precision, simplicity, minimal sample requirement and high sample through-put are the characteristics of an ideal analytical method. Of all currently available techniques for measuring copper in biological samples only one has most, and for some copper-rich specimens, all of these characteristics: this technique is flame atomic absorption spectrometry (FAAS). A reasonably second choice is graphite furnace atomic absorption spectrometry (GFAAS), followed by electrochemical analytical methods such as differential pulse voltammetry (DPV), differential pulse anodic stripping voltammetry (DPASV), and potentiometric stripping. Thereafter, one faces a wide range of analytical techniques with instrumental and methodological complexity which vary to extents such that with the exception of some cases in which these methods are beneficial as reference methods their use appears to be decided more by the partiality or enthusiasm of their protagonists rather than the need to produce reasonably quickly reliable analytical data.

Some complex and expensive techniques which admittedly have advantages of multi-element analysis lack the precision of simpler techniques. Neutron activation analysis (NAA), for example, gave relative standard deviations (RSD's) of 5.7% for serum copper (Versieck et al., 1976) and 5.3% for copper in NBS SRM Bovine liver. Similar precision data were obtained using X-ray emission techniques with typical RSD's of 5.9% for copper in serum at 500 μg/L using photon induced X-ray emission (Kleimola et al., 1980) and of 4.4 % for copper in serum at 1.05 mg/L using energy dispersive X-ray emission (Rastegar et al., 1984). Laser probe mass analysis (LAMMA) may be ideal for the semiquantitative histochemical detection of many elements down to 10^{-18} to 10^{-20} but the precision is poor. It was also reported that copper and a number of other elements (V, Cr, Ni, Co, Mo, Pt, Hg and Bi) in samples of high salt content such as sea water and urine could be determined at μg/L levels by inductively coupled plasma-source mass spectrometry after separation from the salt matrix by chelate complexation (Plantz et al., 1989). Although not recommendable for routine use this might be a valuable reference method for the more practicable GFAAS approach in urinalysis.

Appreciable precision and low bias can be obtained by use of electrochemical analytical methods. Adeloju (1985) determined relatively low levels of copper in muscle, urine and NBS SRM Orchard leaves using this method and higher concentrations in NBS SRM Bovine liver using differential pulse polarography (DPP). All results were within the certified ranges and the precision ranged from 1.9% to 3.1%. The main limitations of this method are the time consumption if several elements have to be determined and the need for a

complete decomposition of the samples. Huiliang et al. (1987) determined mercury, copper and bismuth in urine by computerized flow potentiometry after decomposition with concentrated nitric acid and potassium permanganate. At a copper concentration of 39.6 μg/L the precision for ten analysed samples was 4.2%, which is quite acceptable at this level.

Emission spectroscopic techniques such as inductively coupled plasma optical emission (ICP-OES) and direct current plasma optical emission (DCP-OES) , include the analysis of copper in biological materials (Delves et al., 1983, Roberts et al., 1985). These techniques, with suitable sample preparation, have sufficient low bias and precision for clinical work but are more expensive and more complex than AAS (Herber et al., 1982). Flow injection-ICP-OES will be mentioned below.

Atomic Absorption Spectrometry

The choice between flame atomic absorption spectrometry (FAAS) and graphite furnace AAS (GFAAS) will be determined by nature of the specimen. For example FAAS is ideal for serum copper determinations in which concentrations are normally between 12 and 26 μmol/L but GFAAS is needed to measure the concentrations of 0.1 to 0.8 μmol/L normally found in urine. It should, however, be noted that FAAS may be used as a rapid screening test for Wilson's Disease with which urine copper levels are usually greater than 3 μmol/L and can often exceed 10 - 20 μmol/L.

Earlier FAAS techniques for measuring serum copper levels which included protein precipitation with trichloroacetic acid (Olson and Hamlin, 1968) and/or solvent extraction have been superseded by simpler procedures using either large sample dilution or viscosity adjusted reference solutions with minimal dilution. The analyses are made mainly using continuous nebulization, discrete sample injection or by flow injection techniques with little advantage gained from flame adaptors to increase sensitivity.

Flame Adaptors and Micro Sampling Devices in FAAS

Watling and Villiers (1971) increased the analytical sensitivity of FAAS by directing the flame gases into a slotted quartz tube, constructed with exit slot at 120° to the entrance slot and mounted onto a burner head. The increase in sensitivity stems from increased residence time of the atomic species in the optical path, it can be up to threefold for volatile elements but is only 1.2 to 1.6 fold for copper in aqueous solutions or in a diluted serum matrix (Bahreyni-Toosi et al., 1984; Brown and Taylor, 1984). The marginal improvement is partly offset by the slight deterioration in precision, increased wash-out time to reduce the carry-over effects and limited off-time of the quartz tube. Therefore, this device offers little advantage over conventional FAAS for analysis of copper in biological samples.

The platinum loop-nickel absorption tube technique of White (1969) gave a 2-fold increase in FAAS sensitivity for copper in aqueous solutions. A later modification of this technique (Hartley and Ellis, 1972) allowed nanogram amounts of free copper to be

measured in plasma following electrolyte separation. Ward et al. (1974) obtained excellent agreement between a modified "Delves cup" micro method and conventional FAAS analysis for copper in plasma of 1 x 10^{-10} g Cu/0.004 A.This technique was only 1.6 times better than FAAS using 100 μL injection volumes, similar to that observed for the slotted quartz tube technique. These techniques, however, have not found widespread use for analysis of copper in biological materials because the increase in sensitivity over FAAS is only marginal.

Continuous Nebulization

Of the two general approaches to sample preparation, relatively large sample dilution or viscosity adjusted standards, one of the authors (H.T.D.) prefers the former. It can easily be shown that using 6% v/v butan-1-ol as diluent, 1 + 9 dilutions of sera produce matrix matched calibration graphs that exactly parallel those of similarly diluted aqueous standard solutions and that hundreds of diluted sera may be analysed without risk of partial nebulizer blockage. Standards containing added glycerol (usually 22% m/v) to match viscosity to minimally diluted (1 + 3) sera may not produce matched matrix effects since droplets size distribution in the nebulised aerosol depends upon surface tension as well as viscosity and protein concentrations markedly affect surface tension. Atomization rates will also be influeneced by protein as well as inorganic constituents. For these reasons a sufficiently large sample dilution to minimise matrix effects is preferred.

In one of the authors' (H.T.D.) opinion the best published method for determining copper (and zinc) in serum using continuous nebulization FAAS is that of Meret and Henkin (1971). It is simple, robust, precise and has a high sample throughput. Since 1971, this method has been used in the author's laboratory for routine analysis of more than 15,000 sera. The only changes made to the original published method were to increase the dilution from 1 + 5 to 1 + 9, to enable smaller samples to be used for paediatric studies (200 μL sample + 1800 μL diluent) and to omit the NaCl additions to the reference solutions. With a nebulizer uptake rate of 3.5 mL/min it is possible to make duplicate determinations of both copper and zinc from 1.0 mL diluent (i.e. 100 μL serum). The method has proved to have a low bias in external quality assessment programmes. The precision (RSD) for both Meret and Henkins original work from the authors laboratory are shown with other data in Figure 1.

Salmela and Vuori (1984) added glycerin (22% m/v) to aqueous reference solutions for viscosity matching and also Na, K, Ca, Mg to compensate for pronounced matrix effects at the lower, 4.33 fold dilution of sera presented to the instrument for FAAS. They report the absence of any nebulizer clogging in 2 years (5,000 samples) work. Their method is accurate in showing good agreement for the determination of reference materials. The within batch precision at 1010 μg/L copper in serum was 3.2%; good but approximately double that obtained with the modified Meret and Henkin procedure (Figure 1). There is clearly little to choose between the performances of the method of Salmela and Vuori and that of Meret and Henkin. In the author's view the greater simplicity of the latter and its better precision make it the method of choice.
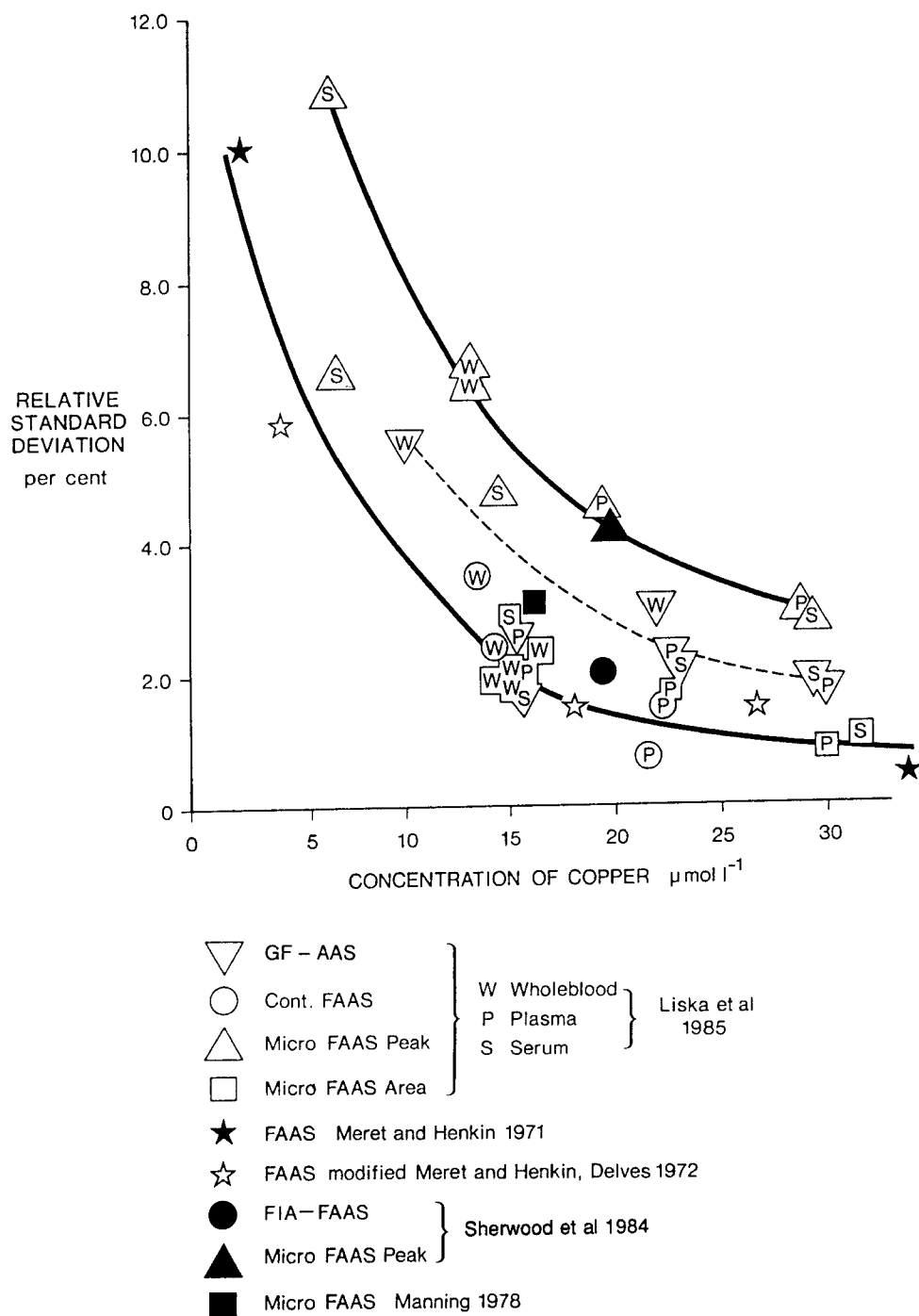
Fig. 1. Precision of the determination of copper in serum as a function of the concentration.

The German "Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Working Group Analytical Chemistry" has developed a continuous nebulization FAAS method for copper in serum based on a 1 + 1 dilution (dilution depends on the applied burner system) with a determination limit of 0.1 mg/L. At a mean copper concentration of 1.24 mg/L the within batch precision was 2.4% and the day-to-day precision 3.2% (Winter and Schaller, 1985). Accuracy was checked against certified reference materials. The method was applied on fortified samples from a serum pool of healthy persons and compared by a GFAAS method developed and tested at the same time (Angerer et al., 1985). The FAAS method is still in successful routine use in a number of German laboratories because of its reliability, simplicity and speed (Schaller, 1993).

Pulsed Nebulization FAAS

Manning (1975) was the first to exploit the observations that small sample volumes (100 μL) could be used for peak absorption measurements by FAAS with negligible loss in sensitivity. He applied this to the determination of copper in serum by injecting 100 μL sample volumes into PTFE cone coupled directly to the capillary of a nebulizer burner and obtained an RSD of 3% at 15.7 μmol/L. With undiluted serum there is obviously a strong risk of blocking the nebulizer capillary and calibration is difficult.

Makina and Takahara (1991) experienced no nebulizer blockage with routine determination using 100 μL injection volumes of a 1 + 5 dilution of serum. Using peak absorbance measurements they obtained RSD's between 1.4% and 3.4% for the concentration range 680 - 2,400 μg/L of copper and their analyses agreed very well with those obtained using much larger volumes and protein precipitation with trichloroacetic acid. The transient peak life was 3 s and the analysis rate was 10 s^{-1}.

Weinstock and Uhlemann (1981) used a commercially available micro injection FAAS system for routine serum copper analyses. Calibration was effected using standard additions to a pooled serum and was linear up to 6 mg/L. The within run RSD's were 1.8% for 100 μL volumes and 2.3% for 50 μL volumes of serum with a copper concentration of 1.2 mg/L. The mean recovery of copper added to serum was 101.5% (range 98.5 to 103%). They obtained excellent data both for the analysis of control sera, and for a comparison of data obtained using a 1 + 3 dilution of sera (r = 0.988). No nebulizer blockage was experienced even with analysis rates of 240 per hour (25 s^{-1}) using a wash solution of aqueous Brij 35 (1 mL/L) between measurements and once per week clean of all equipment coming in contact with the sera, in a solution of 20 g/L pepsin in dilute hydrochloric acid.

Flow Injection or Controlled Dispersion Analysis with FAAS and ICP-OES

The next logical step in improving micro FAAS procedures was to use discrete sample injections into a flowing carrier liquid being pumped into the nebulizer of a flame atomic

absorption instrument. The dilution or dispersion of the sample is determined by the sample volume, flow rate of the carrier liquid and the dimensions of the tubing.

Early clinical studies of flow injection techniques for measurement of copper (and zinc) by Rocks et al. (1982) gave an excellent agreement with a routine FAAS method using TCA precipitation with FIA = TCA - x1.02 - 0.54) μmol/L. This manual injection FIAS system gave an RSD of 2.12% at 19.6 μmol/L, provided the injection rate was carefully controlled. The authors then developed their controlled dispersion analysis (CDA) system (Sherwood et al., 1984) in which the volume selected by an autosampler was controlled (but continuously variable) using a stepping motor and the sample was then injected at a controlled rate into a flowing carrier liquid. This simple system enabled the same manifold to be used for a wide variety of specimens. The dispersion for analyses of copper in serum with 120 μL volumes was 1.7 and the within batch precision was 0.96%.

Flow injection with FAAS was studied for the determination of copper, zinc, and iron in parotid saliva of normal subjects by Burguera et al. (1986). The sample was injected as a discrete slug into a carrier stream of double distilled and deionized water, and standards were prepared in solutions containing physiological concentrations of sodium, potassium and albumin. Sample size for copper determination was 50 μL, dispersion tube length 60 cm and the carrier flow rate 1.5 mL/min. At a mean copper concentration of around 70 μg/L the within batch precision was 2.2% (RSD) and the day to day precision 3.9% (RSD).

McLeod et al. (1984) described the simultaneous determination of Na, K, Ca, Mg, Li, Cu, Fe and Zn in blood serum by flow-injection analysis - inductively coupled plasma optical emission spectrometry (FIA-ICP-OES) using aqueous synthetic multi-element solutions for calibration. With an injected volume of 20 μL the performane data for copper in an 1 + 1 diluted serum ranged for precision from 1.1% to 2.2% with a detection limit of 0.01 mg/L.

Graphite Furnace AAS

Except for extremely low concentrations of copper, e.g. urine or for tissues that are not suitable for direct nebulization e.g. seminal plasma, there is no practical advantage in using GFAAS rather than FAAS for the analysis.

Carelli et al (1982) have described a fairly rapid method for the determination of copper in urine by GFAAS. Standard additions were used to to overcome the small but nonetheless significantly variable suppression of analyte sensitivity by the urine matrix. Sample preparation was simply a 1 + 4 dilution with water. The detection limit using 50 μL injection volumes was 0.2 μg/L and RSD's were less than 3% over the range 10 - 2,000 μg/L. A similar method but using smaller injection volumes, 10 μL, has been in use in one of the authors' (H.T.D.) laboratory for some years (Bunker et al., 1984). The disadvantage of standard additions is offset by the reliability and simplicity of the method.

Pleban and Mei (1983) measured copper in seminal plasma at 30 - 200 mg/L using Zeeman GFAAS following a simple 1 + 4 dilution in water. Calibration was achieved using matrix based reference solutions. Spermatozoa were analysed following separation by centrifugation, lyophilization and digestion with nitric acid. The RSD's were 5% at 147 μg/L

and 2% at 1.81 mg/kg. The limited number of cells available for analysis, matrix viscosity and relatively low copper concentrations precluded the use of FAAS for these measurements.

Angerer et al. (1985) developed a GFAAS method for the determination of copper in serum and urine. The samples were directly injected into the graphite tube. The injected volume was 10 μL for serum and 50 μL for urine. Calibration was done against matrix matched samples. Within run precision for serum with 1 mg/L copper concentration was 2.3%, for urine in the range of 20.8 to 126.6 μg/L from 2.3% to 4.1%. Accuracy was tested with reference samples and by the FAAS method developed at the same time (Winter and Schaller, 1985). Under the given conditions the detection limit was 0.015 mg/L for serum and 3 μg/L for urine.

Dube (1988) described a method for the direct determination of copper in urine and whole blood based on Zeeman-effect GFAAS with automated injection. Except for a dilution step, no sample preparation was required. Injected volumes were 10 μL of blood (1 + 50 diluted) and 20 μL (1 + 1 diluted) of urine. Calibration was done against a matrix matched calibration graph from which all calculations were made in a single analytical run. The detection limit calculated for undiluted urine was 0.75 μg/L, within-batch and between-batch precision ranged from 2 to 8% for urine samples in the range of 4-65 μg/L Cu. Precision for blood was at the same order as for urine. The time per analysis (including the furnace cooling time) was about one minute. Accuracy and bias of the method were evaluated by internal and external QC samples, as well as by interlaboratory comparisons.

Comparison of FAAS and GFAAS for Analysis of Copper in Biological Samples

Liska et al. (1985) made an excellent assessment of the relative merits of GFAAS and three different modes of FAAS for the analysis of copper in whole blood, in plasma and in serum. The GFAAS procedure was at best 5 times slower than FAAS methods and the precision of 1.7% to 5.4% (RSD) was generally worse. Precision data from this study are given in Figure 1, together with data from Meret and Henkin (1971), from observations in the authors (H.T.D.) laboratory using a slightly modified version of these latter authors method, from Manning (1975) and from Sherwood et al. (1984). The lower continuous curve in Figure 1 shows the variation of precision with copper concentrations in whole blood, serum or plasma using either continuous nebulization FAAS (Liska et al., 1985; Delves, 1985; Meret and Henkin, 1971) or pulsed nebulization with integrated absorption measurements (Liska et al., 1985). The RSD pulsed for nebulization FAAS obtained by Manning (1975) using peak height measurements and the FIA-FAAS precision data from Rocks et al. (1984) using manual injection lie above this line whereas, the precision of CD-FAAS, 0.96 reported by these latter authors lies below this line. The upper continuous line shows the poorer precision observed for pulsed nebulization FAAS and peak height measurements (Liska et al., 1985; Sherwood et al., 1984). The dotted central line describes the precisions obtained by the former authors using GFAAS with manual injections. It is possible that somewhat better precision would have been obtained using an autosampler.

It is both interesting and pleasing to note that despite considerable improvements in atomic absorption instrumentation over the past 20 years, the analytical precision reported by Meret and Henkin in 1971 has not been substantially improved. This shows clearly that simple methods may produce precise results.

Most currently available methods have RSD's that lie close to the lower continuous line, e.g. Weinstock and Uhlemann (1981) with 1.8 % at 1,200 μg/L using pulsed nebulization FAAS and Velghe at al (1982) with RSD's of 1.2% - 2.1% at 890 - 1,540 μg/L using direct GFAAS measurements in 1 μL volumes of serum.

At copper concentrations below 5 μmol/L where the precision data in Figure 1 became just acceptable, the more sensitive GFAAS can be used with advantage. For instance Carelli et al. (1982) reported an RSD better than 3% for copper in urine down to 0.2 μmol/L. However, at higher concentrations the higher sample throughput of FAAS is more attractive than the slow rates encountered with GFAAS methods.

All of the authors cited here have demonstrated a good degree of analytical accuracy and/or bias for their methods by comparison with other established techniques by performances in external quality assessment programmes, or by analysis of reference materials (CRM's) certified for its copper concentrations. For the CRM's examples are listed in the chapter on Reference Materials. It is difficult to assess from these data the relative accuracies of the procedures but if, as is likely, they are all comparable, as are their analytical precisions then the deciding features must be speed of analysis and availability of instrumentation. Therefore, for most applications FAAS in one mode or another would still be the method of choice.

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Chapter 18

Lead

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INTRODUCTION

Lead may be determined in a variety of biological materials such as blood, urine, soft tissues, teeth, bones, hairs, leaves and wood. From the viewpoint of occupational and environmental toxicology the determination of lead in blood is of greatest importance, since the concentration of lead in whole blood (PbB) is considered to be the best indicator of current lead exposure in humans and mammals (Alessio et al., 1979; Ewers et al., 1991; Lauwerys, 1975; Vahter, 1982; WHO, 1977, 1980). Therefore, the problems of PbB determination are broadly discussed in this chapter, whereas the techniques and problems of lead determination in other biological matrices such as teeth, bones, plant and animal tissues are reviewed to a minor extent.

DETERMINATION OF LEAD IN WHOLE BLOOD (PbB)

General remarks: Assessment of lead exposure

The determination of PbB is of prime importance with respect to the diagnosis of lead poisoning and to the assessment of hazardous conditions both in occupationally exposed people and in the general population. It is generally agreed that the concentrations of lead in air, food and water are less relevant for assessing health hazards for humans than the amount of lead actually absorbed and this is what actually is reflected by PbB (WHO, 1977, 1980).

In interpreting PbB levels one must consider the fact that they reflect a dynamic equilibrium between exposure (intake and absorption), distribution, and elimination. Under

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steady state conditions, e.g. those prevailing in the general population or during long-term, unchanged occupational exposure, the PbB level gives a good picture of current exposure, but shortly after changes in exposure intensity the level of PbB becomes a poorer indicator. For example, after start of occupational exposure it takes about two month for the PbB level to reach a steady state. After the termination of exposure the PbB level decreases slowly with a half-time of 2 - 4 weeks (Lauwerys, 1975; WHO, 1977, 1980).

Blood contains lead in three forms: a major fraction (about 95%) bound to erythrocytes, a protein-bound fraction in plasma, and a diffusible fraction that represents the metabolically active form of circulating blood. Since the major fraction of PbB is bound to erythrocytes, some authors have proposed expression of results in μg/100 mL of erythrocytes so that variations in the hematocrit value are taken into account. However, the biological validity of this correction is still controversial. With the improvement of the analytical sensitivity and reliability of the methods for measuring low levels of lead in biological materials, it is likely that, in future, the concentration of diffusible lead in plasma as an index of metabolically active lead will be further evaluated (Lauwerys, 1983).

Kinetic studies in man show that the lead body burden consists essentially of three compartments: (1) a rapid exchange pool in blood and soft tissues; (2) an intermediate exchange pool in muscles, skin and bone marrow; (3) a slow exchange pool in dense bones and teeth (biological half-life about 20 years). The amount of lead stored in the latter compartment increases throughout life (Steenhout, 1982; Steenhout and Pourtois, 1981).

Past exposure to lead, which is not necessarily reflected by elevated PbB levels, can be estimated by measuring the amount of lead excreted in urine after provocation with a chelating agent, e.g. EDTA (Alessio et al., 1979). If, after administration of 1 g EDTA, the amount of lead in urine exceeds 1 mg in 24 h, the test usually is considered positive (Lauwerys, 1983). Measurement of lead in hair allows the estimation of exposure during the previous months. Although hair is an easily available material, this method may not be reliable because it is highly difficult to distinguish between lead incorporated into the hair and that simply adsorbed on its surface.

The lead body burden can be assessed by determining the lead concentration in deciduous (children) or permanent (adults) teeth. In recent years this approach has attracted increasing interest and a number of studies on tooth lead levels have been published, using in most cases shed deciduous teeth of children living in different environments (Ewers et al., 1990; Fosse and Justesen, 1978; Grandjean et al., 1984, 1986; Mackie et al., 1977; Shapiro et al., 1972, 1978). Since the formation of deciduous teeth begins in prenatal life, the average lead level in the shed teeth may be taken as an integrated measure of the total exposure during early life. In adults the tooth lead levels increase with age parallel to the accumulation of lead in dense bones (Steenhout and Pourtois, 1981; Steenhout, 1982).

The PbB level as an index of the absorbed amount of lead depends on a variety of environmental factors (lead intake via air, food, water, and dust; solubility of the lead compounds ingested or inhaled, composition of the diet) as well as biological factors (age, sex, iron deficiency). Due to a greater food consumption, males have, on average,

slightly higher PbB levels than females.

Since the determination of lead in urine does not require blood sampling, it is some-times preferred over PbB-determination. Lead in urine is dependent on the PbB level and thus reflects the amount of lead recently absorbed. However, it is influenced by several factors such as fluid intake and renal function and, therefore, may not be a reliable indicator of exposure (Lauwerys, 1983; WHO, 1981).

Analytical problems of PbB determination

PbB-determinations can be subjected to a number of significant analytical errors. This is true especially for the past, where many laboratories performed the determinations unsatisfactorily. *Several reports on interlaboratory comparative studies (Boone et al., 1979; Lauwerys et al., 1975; Maher et al., 1979; Parsons, 1992)* showed that the results obtained by different laboratories differed significantly, in some cases by a factor of up to 10. PbB levels reported in older studies, therefore, have to be considered cautiously and many discrepancies with regard to dose-effect relationships arise from comparison of incompatible results.

The poor performance of laboratories providing analyses of PbB led to the establish-*ment of proficiency testing programs for PbB determinations* on the regional, national and international level. For example, the Health and Safety Directorate of the Commission of the European Communities organized a quality control program within the framework of the biological monitoring campaign for PbB according to the "Council Directive on Biologi-cal Screening of the Population for Lead" (Berlin et al., 1973). The program was started in 1979 and included 33 laboratories. A similar quality control program including laboratories in 10 different countries was carried out within the UNEP/WHO Pilot Project on "Assess-ment of Human Exposure to Lead and Cadmium Through Biological Monitoring" (Lind et al., 1987; Vahter, 1982; Vahter and Friberg, 1988). Long-term proficiency testing pro-grams for PbB analyses, which are currently in operation, were established in Canada, Germany, Great Britain, Italy, the United States and some other countries. Reports on the performance of laboratories participating in these programs are available in the literature (Bullock et al., 1986; Morisi et al., 1989, Parsons, 1992; Schaller et al., 1987).

In general, the results of these proficiency testing programs indicate a considerable improvement in the performance of PbB determination in most laboratories (for review see Parsons, 1992). According to the practice in clinical chemistry, quality assurance including internal and external quality control should, therefore, be an integrated part of PbB measurements.

In many countries PbB determination in lead-exposed workers may only be performed by laboratories which hold a licence stating their successful participation in proficiency testing programs (OSHA, 1982; Parsons, 1992; Schaller et al., 1987). Proficiency testing is conducted 1 - 4 times a year or even more often. In some countries licensed laboratories must also submit to an on-site inspection of their facilities and be staffed with appropriately qualified personnel.

Quality control in PbB determinations

The correct performance of PbB determination must include (a) the correct sampling of blood (preanalytical quality control) and (b) an internal and external quality control within each set of PbB determinations.

Blood sampling and preanalytical quality control

There are many possibilities to contaminate biological samples, e.g. through the use of unsuitable blood collection vials and contaminated anticoagulants. Furthermore, contamination may originate from the skin if not properly cleaned or from contaminated cleaning solutions. Studies on commercially available, disposable syringes and needles have shown that diluted nitric acid as well as blood may extract lead and other metals from vials and syringes in quantities, which would invalidate any measurement of these metals within the normal concentration range.

To avoid as much as possible contamination of the blood samples the following means should be taken into consideration:
1. The syringes, needles and collection tubes used should be checked previously to be free of contamination by lead. Practically, it is sufficient to check some randomly selected vials or tubes from the same lot. Subsequently, only vials and tubes from this lot should be used.
2. The skin should be carefully washed and then cleaned with disposable napkins saturated with a desinfectant (usually 70% isopropyl alcohol). The napkins and the desinfectant should be checked for metal content previously.
3. If the blood sampling is not performed by specially trained personnel, it is useful to prepare a written instruction for the sampling of blood. If possible, it might be advantageous to provide demonstrations and training for the personnel collecting the blood.

Quality control during PbB determinations

A rigid quality assurance scheme including internal and external quality control, the control of blanks and the use of control charts should be an integrated part of all PbB determinations (for details see WHO-Euro, 1981). Any change in the procedure of determination as well as the introduction of new standards or control samples should be recorded. Likewise, any changes in instrumentation or personnel should be recorded.

All laboratories performing PbB determinations should participate in at least one proficiency testing program for PbB. As mentioned above a number of agencies and scientific organizations are performing regular quality control programs for PbB analyses as a part of proficiency testing for occupational and environmental toxicology laboratories. Table 1 presents an overview on some of these programs and the acceptance criteria applied. The dispatched external quality control samples usually cover at least two different PbB levels, one in the range of general population lead exposure and one in the range of occupational lead exposure.

TABLE 1

ACCEPTANCE CRITERIA APPLIED IN VARIOUS PROFICIENCY TESTING PROGRAMS FOR PbB ANALYSES (ADOPTED FROM PARSONS 1992) AND SUPPLEMENTED).

Proficiency testing program	Criteria	100 μg/L range
U.S. Public Health Service, Centers for Disease Control (CDC)/Health Resources Services Administration (HRSA)	$\pm$ 40 μg/L if value is < 400 μg/L $\pm$ 10% if value is $\geq$ 400 μg/L	6-14
U.S. Occupational Safety and Health Administration (OSHA)	$\pm$ 60 μg/L if value is < 400 μg/L $\pm$ 15% if value is $\geq$ 400 μg/L	4-16
State of New York, State Department of Health	$\pm$ 40 μg/L if value is < 400 μg/L $\pm$ 10% if value is $\geq$ 400 μg/L	6-14
Wisconsin State Laboratory of Hygiene		
Commonwealth of Pennsylvania, Department of Health		
American Association of Clinical Chemistry (AACC)/College of American Pathologists	+ 60 μg/L if value is < 400 μg/L + 15% if value is $\geq$ 400 μg/L	4-16
U.K. External Quality Assessment Scheme for PbB analyses	Depends on variance index score	
Centre de Toxicologie du Quebec, Canada	$\pm$ (0.04 x + 2.6)	7-13
Deutsche Gesellschaft für Arbeits- und Umweltmedizin	$\pm$ 10% at levels around 400 μg/L	
Commission of the European Communities	$\pm$ (0.04 x + 2.6)	7-13

In the past, a major problem associated with the evaluation of results was the lack of reference materials with assigned or certified values. A number of reference materials with certified lead levels are now available (see Chapter 11). In general, these reference materials consist of lyophilized blood. The samples have to be reconstituted by addition of deionized water according to the instructions and should be used for evaluating the bias of the lead determinations.

Blood samples dispatched within the framework of proficiency testing programs should not be used for internal quality control purposes since most are designed for short-term evaluation purposes and are not stable enough for long-term use. Moreover, in most cases target values are made available long after the samples have ceased to be of any use.

The need for increased precision and lower bias in the determination of low levels of PbB has been emphasized in some recent publications (Flegal and Smith, 1992; Parsons, 1992). This is due to the fact that, in the United States, the PbB level of concern for early

toxic effects was recently lowered from 250 μg/L to 100 μg/L (CDC, 1985, 1991). Moreover, there has been a significant decline of PbB levels in the general population of many countries to average values well below 100 μg/L. It has been recognized that the bias and precision of many routine PbB determinations below 100 μg/L are still inadequate.

Methods for PbB determinations

The literature on procedures for PbB determination is abundant. Those techniques that have been shown to provide accurate and precise PbB determinations in routine use include anodic stripping voltammetry (ASV), flame atomic absorption spectrophotometry (FAAS), discrete sampling FAAS, and graphite furnace AAS (GF-AAS). The method most widely used for routine determination is AAS in its various modifications. The relatively slow analysis rate of ASV tends to limit the application of this technique to that of a backup or reference method. Whatever the technique which is applied, it should be emphasized that avoidance of contamination, careful handling of the blood samples and frequent intra- and interlaboratory checks are more important for ensuring precision and reliability than the method itself.

Subsequently four procedures based on AAS determinations are described, which have proved to be valid and reliable in experienced hands. The AAS determinations generally are performed at a wavelength of 283.3 nm.

Extraction method for flame AAS determination of PbB

This procedure was originally developed by Lehnert et al. (1969) and has been adopted as an officially recommended analytical method for PbB determination in blood and urine by the working group "Analytical Chemistry" of the "Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area" of the Deutsche Forschungsgemeinschaft in 1974 (Angerer and Schaller, 1985; Henschler). The method can be described as follows:

1. Pipet 2 mL of heparinized blood into a lead-free 10 ml plastic tube.
2. Pipet 0.1 mL of Triton X-100 solution (Triton X-100 diluted with water 1 + 1, v/v) and 0.5 mL of a 10% solution of ammonium pyrrolidin dithiocarbamate in water into the tube.
3. Homogenize 30 s at 22 000 rpm.
4. Pipet 1 mL of methyl isobutylketone into the tube and homogenize again for 2 min.
5. Cap the tube and centrifuge for 3 min at 2000 - 3000 g.
6. Pipet the organic phase into another acid-washed vial, from which it is aspirated into the burner.

Dilution method for GF-AAS analysis of PbB

This method was first described by Fernandez (1975, 1978) and has been adopted as an officially recommended analytical method for PbB analysis by the working group

"Analytical Chemistry" of the "Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area" of the Deutsche Forschungsgemenschaft (Henschler). Since GF-AAS analysis of diluted blood is strongly influenced by matrix effects it is recommended to use the internal standard addition method for calibration. For routine analyses, however, calibration may also be performed by the use of lead-spiked blood. The method subsequently described is based on the internal standard addition method:

1. Pipet aliquots of 50 μL of EDTA blood into four acid-washed 1500 μL Eppendorf centrifuge tubes containing 850 μL of 0.1% Triton X-100. Flush the pipet tip 3-4 times with the Triton X-100 solution to minimize transfer error.

2. Pipet into tube 1 100 μL of water, into tube 2 100 μL of standard solution 1 (2000 μg/L Pb), into tube 3 100 μL of standard solution 2 (4000 μg/L Pb), into tube 4 100 μL of standard solution 3 (6000 μg/L Pb). The standard solutions 1 - 3 are prepared by diluting the stock solution with 0.01 M HNO_3. To make a chemical blank pipet 150 μL of water and 850 μL of 0.1% Triton X-100 into an Eppendorf centrifuge tube.

3. Cap the tubes and shake them well to obtain equilibration.

4. Measure the lead content of the solution by GF-AAS (injection volume: 20 μL).

The temperature-time program of the GF-AAS analysis has to be optimized for the apparatus used and the matrix to be analyzed by the analyst. The working conditions given in Table 2 may serve as orientating guidelines.

The detection limit of the method is given to be 20 μg/L of whole blood.

TABLE 2

TEMPERATURE-TIME PROGRAM FOR PbB DETERMINATION BY THE TRITON X DILU-
TION METHOD (dependent on instrument used)

Step	Ramp time (s)	Hold time (s)	Temperature (°C)
Drying	20	20	125-140
Charring	25	25-60	490-560
Atomization		5	2100
Burning out		5	2300

Deproteinization method for GF-AAS determination of PbB

This method was developed by Stoeppler et al. (1978). It has the advantage of a simple and fast sample pretreatment. Since lead is determined in an acidic aqueous solution obtained from blood by acid deproteinization, the matrix influences are relatively low and there are practically no salt residues left in the graphite tube. The method,

TABLE 3

TEMPERATURE-TIME PROGRAM FOR PbB DETERMINATION APPLYING THE DEPROTEINIZATION METHOD ACCORDING TO STOEPPLER ET AL. (1978) (dependent on instrument used)

Phase	Ramp time (s)	Hold time (s)	Temperature (°C)	Gas flow (ml/min)
Drying	2	15	90	300
Drying	20	5	130	300
Charring	15	40	400	300
Setting gas flow	1	15	400	0
Atomization	1	6	2200	0
Burning out	1	3	2700	300

therefore, is well suited for large series of PbB determinations. A further advantage is that cadmium can be determined from the same solution. It is recommended to use EDTA blood since the addition of heparin to the blood yields a loss in sensitivity. The procedure (slightly modified for practical reasons) is as follows:

1. Pipet 200 μL of whole blood into a 4 mL acid-washed polystyrene tube. To make a chemical blank 200 μL of water are pipetted instead of blood. If the calibration is performed by the internal standard addition technique, it is important to pipet exactly 200 μL of blood. This may achieved by the "reverse pipetting technique".
2. Add 800 μL of 1 N HNO_3 to the blood.
3. Cap the tubes and shake them by means of an horizontal electrical shaker for 30 s.
4. Centrifuge for 10 min at 4000 rpm.
5. Transfer 400 μL of the supernatant into acid-washed polyethene sups placed in the tray of an automatic sample dispenser (e.g. Perkin-Elmer AS1 or AS40).

The temperature-time program, which in the authors' laboratory has been proved to be well suited for Pb-determination in the supernatant is given in Table 3. These working conditions may only serve as guidelines and have to be optimized by each analyst for his/her special conditions.

Calibration is carried either by means of internal standard addition or against a calibration curve from lead-spiked blood. For routine purposes the much simpler and faster method of calibration with lead-spiked blood can be applied. To minimise matrix effects the use of Zeeman GF-AAS and pyrolytically coated graphite tubes with a L'vov platform is recommended, but this is instrument-dependent.

The lead-spiked blood should be prepared in the following way:

1. Human blood with a low lead content is, under continuous stirring, divided into four aliquots of 99 mL (104 g) using acid- washed polyethene bottles with screw caps.

2. Prepare the following standard solutions by diluting the stock solution with 0.01 N HCl/0.9% NaCl: S1: 15 mg Pb/L; S2: 30 mg Pb/L; S3: 60 mg Pb/L. S0 consists of 0.01 N HCl/0.9% NaCl, which has been used for the dilution of the stock solution. Pipet 1 mL of the standards S0 - S3 to the aliquots of blood under continuous stirring and keep stirring the mixture at 37°C for 2 h to obtain equilibration. By this procedure S1 is spiked with 15 μg/100mL, S2 with 30 μg Pb/100 mL, and S3 with 60 μg Pb/100 mL. The lead content of the blood used for the preparation of spiked blood specimen is determined by the internal standard addition method and has to be taken into account in the evaluation.

3. The batches of S0 - S3 are divided into 5 mL aliquots, which are stored at -20°C. Every day one aliquot of S0 - S3 is thawed and used for calibration in such a manner that S0 - S3 are measured before and after a set of about 10 - 15 blood samples on the tray. If no significant drift effects occur the calibration graphs obtained from one tray can be combined and used for calibration. The calibration curve is linear up to about 400 μg/L (including S2, if the blood used for the preparation of the standards contains less than 100 μg/L).

Delves cup technique for PbB determination

The so-called Delves cup method was applied in many laboratories for PbB determination before the graphite furnace technique reached its highly developed present stage. The method was published by Delves (1970) and at that time it was one of the first reliable micro methods requiring only very small amounts of blood. It has been adopted by several authors for the analysis of capillary blood as well as for lead determinations on disks punched from filter paper previously spotted with a drop of blood (Cernik and Sayers, 1971; Cernik, 1974; Delves, 1977).

In principle, 10 μL of whole blood are transferred into a 10 x 5 mm nickel crucible and dried on a hot-plate at 120 - 140°C (about 30 s) while taking care to avoid any spitting of the sample. After cooling one pipets 20 μL H_2O_2 into the crucible, which then is placed again on the hot-plate for 1 - 2 min until a dry creamy yellow residue is obtained. Subsequently the cup is placed in the holder of crucible sampling unit (for details see Cernik, 1974) and introduced into the air-acetylene flame of a three-slot burner. In order to increase the sensitivity of the measurement a nickel absorption tube is placed a few centimeters above the burner. The light beam passes through the absorption tube, which has to be adjusted previously for minimum light loss. The speed with which the crucible is inserted into the flame is not critical, but it should be sufficiently rapid to prevent pre-combustion of the samples. The absorption is recorded at a wavelength of 283.3 nm. The crucibles are cleaned when heated in the flame during analysis and are ready for further determinations without any additional treatment.

The calibration graph is constructed by using the above procedure, adding 10 1nm1bL volumes of lead standard solutions containing 0, 10, 20, 40 and 800 μg/L Pb to crucibles containing 10 μL volumes of blood with a low lead content. The standard curve has to be *parallelly moved to zero before evaluation of the samples.*

The precision of the method, as reported by Delves (1970) and expressed as relative standard deviation, is about 15% at PbB levels around 100 μg/L and 4 - 6% at PbB levels in the range of 300 - 800 μg/L. The lower limit of detection is 12 μg/L Pb in blood.

DETERMINATION OF LEAD IN TEETH (PbT)

In performing tooth lead analysis, it has to be considered that the lead concentrations vary among different tooth types (when analyzing whole teeth) as well as, within one tooth, between different tooth tissues (Ewers et al., 1990; Grandjean et al., 1984, 1986). In deciduous teeth the lead concentration tends to decrease from the medial incisors to the premolars. Whether there is a similar variation in permanent teeth of adults has not yet been evaluated. Thus, when comparing analytical data on lead concentrations in whole teeth, it should be stated what type of tooth was analyzed.

Within a tooth the highest lead concentrations are found in the inner and outer surface of the tooth, i.e. in the outer layer of the enamel and in the circumpulpal or secondary dentine (Grandjean et al., 1984; Shapiro et al., 1972, 1978). The circumpulpal dentine, which is formed after eruption of the tooth, continues to accumulate lead until the tooth is shed or extracted. It has been claimed that the lead concentration in the circumpulpal dentine reflects the integrated lead exposure during the time from completion of tooth formation until tooth extraction or shedding in a better way than does the average lead concentration in the whole teeth (Grandjean et al., 1984). However, other authors Ewers et al. (1990), Fosse and Justesen (1978), Mackie et al. (1977) have recommended the analysis of whole teeth, since the procedure is simple and does not require special preparation of deciduous teeth begins in prenatal life; thus, the average Pb-level in the tooth as a whole could be regarded as an indicator of the total lead exposure during early (i.e. pre- and postnatal) life.

The analytical techniques used for tooth lead analyses include AAS, ASV, and X-ray fluorescence analysis. The X-ray fluorescence technique allows the measurement tooth of lead levels in situ (Shapiro et al., 1978). AAS and ASV determinations require the tooth to be dissolved in nitrous acid prior to analysis.

When analyzing teeth for lead significant analytical problems may arise due to the presence of relatively large amounts of the inorganic matrix (mainly calcium and phosphate). Particularly in AAS determinations the matrix effect produces a strong decrease of sensitivity. The difficulty could be overcome by extracting the lead from the aqueous solution, but it proved that the extraction yield is also strongly affected by the tooth matrix. When the ASV technique is applied, the matrix effects appear to be significantly less important.

Due to these analytical difficulties is appears that, particularly in the past, many tooth lead measurements have been performed unsatisfactorily. An interlaboratory comparison program on tooth lead analysis, organized by a IUPAC subcommittee, showed large interlaboratory variations (Stack and Delves, 1982) and in recent years the need for strict quality control has been emphasized repeatedly.

The introduction of pyrolytically coated graphite tubes and the L'vov platform technique in GF-AAS as well as of the Zeeman-GF-AAS technique offer improved possibilities to reduce matrix effects when measuring tooth lead in aqueous solutions. Where these techniques are available calibration with lead-spiked tooth solutions can be applied. Otherwise the analyses must be carried out by means of internal standard addition. The accuracy of the analytical procedure should be assessed by interlaboratory comparison, since, at present, no reference material of human teeth is available.

The procedure subsequently described has been shown to provide accurate and precise analytical data in routine tooth lead analyses in the authors' laboratory.

Pretreatment of teeth:

1. Clean each tooth with warm deionized water and a filter paper to remove residues of blood or tissue.
2. Dry the teeth under an infrared lamp (1 min).
3. Place the tooth in an acid-washed, calibrated 25 mL flask, add 3 ml of concentrated HNO_3, and heat the flask on a hot-plate.
4. Dilute the tooth solution with water to 25 mL.
5. Pipet 200 μL of this solution into an acid-washed polyethene cup placed on the tray of the automatic sample dispenser and add 1000 μL of 0.01 N HCl.

GF-AAS measurement:

AAS (Perkin Elmer 3030) with atomization unit HGA 500 and automatic sampling system AS 40. Graphite tubes with L'vov platform (Perkin Elmer). Injection volume: 20 μL.

TABLE 4

TEMPERATURE TIME PROGRAM FOR PbT ANALYSIS (dependent on instrument used)

Phase	Ramp time (s)	Hold time (s)	Temperature (°C)	Internal flow (ml/min)
Drying	2	15	90	300
Drying	20	5	130	300
Charring	35	10	500	300
	1	10	400	300
	1	3	400	0
Atomization	0	3	1500	0
Burning out	1	4	2700	300
Cooling	1	15	30	300

A calibration graph is constructed from lead-spiked tooth solutions. The lower detection limit of the method is 0.5 mg Pb/kg tooth. The precision expressed as relative standard deviation was found to be 3.2% at a tooth lead concentration of 6.83 mg Pb/kg tooth.

DETERMINATION OF LEAD IN OTHER BIOLOGICAL MATERIALS

The determination of lead and other trace elements in biological materials usually requires the transformation of a solid sample into a homogeneous analyte solution. A variety of methods including dry ashing at high temperature, low-temperature ashing in microwave excited oxygen plasma, wet ashing (either in open or closed systems under pressure) and tissue solubilization with quarternary ammonium hydroxide compounds can be applied. In principle, the determination of lead in biological materials involves the same problems as PbB determination. Usually, a chain of possible error sources exist including sampling, storage, sample pretreatment, the lead determination itself and weight normalization. Each particular step may contribute to contamination and losses as well. Thus a careful evaluation and performance of all steps of the analytical procedure is a prerequisite of reliable analyses. A further prerequisite to obtain reliable results is the application of appropriate reference materials. Simultaneous application of independent analytical procedures to the same material as well as interlaboratory comparisons are also a practical way to obtain accurate data, particularly in those cases where no appropriate reference materials are available.

A number of biological reference materials for trace element analysis are now available to interested laboratories. An overview on those materials is given in some recent publications Berman and Sturgeon (1987), Christensen et al. (1987), Parr et al. (1987), Rasberry (1987).

Finally, it should be pointed out that, in recent years, techniques have been developed to measure the lead content in bones and teeth *in vivo*. The method applied is X-ray fluorescence (XRF), and promises to provide a convenient, non-invasive assessment of body lead burden. The possibilities of this technique are described and reviewed by Hu et al. (1989) and Shapiro et al. (1978).

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Manganese

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INTRODUCTION

Although manganese has been proved to be essential in many animal studies, proof of its essentiality in humans is lacking. In most animal species, manganese deficiency results in skeletal abnormalities, poor posture and impaired reproduction (Leach, 1974). Abnormalities in carbohydrate and lipid metabolism have also been noted. Only one unsubstantiated case of manganese deficiency in humans has been reported (Doisy 1974). Symptoms seen included a decrease in plasma cholesterol; slowed growth of hair and nails; a reddening of the beard and hair and scaly dermatitis. The patient also had vitamin K deficiency at the same time.

Lack of awareness of the analytical problems of sample contamination and determination of very low levels of manganese has led to many confusing claims. Tanaka (1977) reported that epileptic children had a mean serum manganese concentration of 8.5 μg/L which was lower than in healthy children (mean concentration 14.8 μg/L); encouraging results were reported for therapy with manganese. Hoffman (1980) later examined serum manganese levels and found no significant difference between epileptics (Mean normal 2.3 μg/L children; 2.4 μg/L adults) and non-epileptics (2.2 μg/L children; 2.4 μg/L adults). Although these values for normals are six times lower than those of Tanaka (1977), even Hoffman's results are high compared to the "consensus mean" (Versieck and Cornelis, 1980) of about 0.6 μg/L.

A further example of this confusion comes from the claim that serum manganese levels increase after myocardial infarction (Hegde et al. 1961, Kanabrocki et al. 1967). In these studies, values for normal levels are far too high. Lower values were found in the work of Kuhn et al. (1978), who found a rise in serum manganese up to a mean level of 2.2 μg/L at two days following myocardial infarction which then fell to a mean value of 1.20 μg/L at eight days. No indication of special procedures taken to minimize contamination were given in their report. By contrast, the careful precautions taken to minimize contamination by Versieck et al. (1975) in manganese analysis are well documented. They found no

statistically significant difference between 16 patients with myocardial infarction (mean ± S.D.: 0.64 ± 0.10 μg/L) and normals (mean ± S.D.: 0.57 ± 0.13 μg/L). Sequential samples were not taken, but the samples were taken at 2 and 4.5 days after onset of myocardial infarction which should correspond to the period of elevation seen by Kuhn et al. (1978).

These examples illustrate the importance of contamination-free sample collection procedures and reliable determination in eliminating potentially misleading data.

Many trace element deficiencies have been observed in total parenteral nutrition (TPN) when trace element supplements have not been added to the feeding regimen (Fleming, 1989). Manganese deficiency has not yet been reported in patients on TPN; nevertheless manganese is considered important enough to add to trace element supplements. A previously commercially available supplement (Addamel, Kabi-Vitrum, Sweden) supplied 2.2 mg/day. This appeared to be excessive as elevated serum manganese levels resulted and increased urinary excretion was seen (Halls and Fell, 1981). More recently (Ejima et al., 1992), symptoms of parkinsonism were seen in a patient on TPN with the same daily intake of manganese. On cessation of manganese supplementation, the symptoms gradually regressed. A modified supplement is now available (Additrace, Kabi-Pharmacia, Sweden) which contains less manganese (0.28 mg/day).

Exposure to excess manganese leads to effects on the brain and respiratory system (Piscator, 1979). Workers exposed to high airborne levels of manganese oxide dust in, for example, mines or ore-processing plants, have an increased incidence of pneumonia and bronchitis. Once absorbed, manganese can affect the brain leading to a disease resembling Parkinson's Disease. Chronic manganese poisoning is irreversible. Determination of manganese in blood and urine may not be very helpful in assessing exposure or risk. From a study of blood manganese levels in workers exposed for different periods in a Norwegian manganese alloy plant, Tsalev et al. (1977) concluded that the determination of manganese in whole blood or serum had no significance in detecting manganese poisoning. Manganese levels in toenails have been suggested as a better marker of exposure to manganese (Tsalev, 1985). However, higher than normal blood manganese concentrations were found in aborigines living near a manganese ore deposit in the Northern Territory, Australia (Hams and Fabri 1988) and these seemed to be associated with manganism (Cawte and Florence, 1987; Cawte et al., 1987). The mean blood manganese concentration of the group studies was 27.0 μg/L compared to 11.8 μg/L in control subjects.

DETERMINATION OF MANGANESE

Problems in determination

Manganese is present in body fluids and tissues at very low concentrations (Table 1). The risk of contaminating specimens is very great since manganese is a common element found, for example, in dust, stainless steel and on plastic surfaces produced on metal moulds. In addition, the determination of a small concentration of manganese in a high

TABLE 1

MANGANESE CONCENTRATIONS IN BODY FLUIDS AND TISSUES

Measured values for healthy adults where care has been taken to reduce contamination and control matrix interference.[a]

Mean concn.		n	Range (or S.D.)	Reference
Serum (μg/L)				
0.63		12	0.36 - 0.90	Fernandez et al. (1963)
0.59		14	($\pm$ 0.18)	Cotzias et al. (1966)
0.57		50	0.38 - 1.04	Versieck et al. (1974)
0.54		11	0.36 - 0.78	Damsgaard et al. (1973)
0.58		9	0.36 - 0.96	Halls and Fell (1981)
0.89		10	0.68 - 1.20	Casey et al. (1987)
0.88		48	($\pm$ 0.11)	Milne et al. (1990)
0.59		31	($\pm$ 0.17)	Nève and Leclerq (1991)
Whole blood (μg/L)				
10		32	6 - 30	Tsalev et al. (1977)
11		33	3 - 21	Muzzarelli and Rocchetti (1975)
9.0		60	3.9 - 15.1	Pleban and Pearson (1979)
12.2		20	($\pm$ 3.9)	Buchet et al. (1976)
8.4		14	($\pm$ 2.7)	Cotzias et al. (1966)
11.8		15	($\pm$ 3.7)	Hams and Fabri (1988)
10.9		48	($\pm$ 0.6)	Milne et al. (1990)
Erythrocytes				
(μg/kg wet weight)				
15.0		46	8.1 - 36.9	Versieck et al. (1974)
(μg/L)				
16.6		48	($\pm$ 1.2)	Milne et al. (1990)
Urine (μg/L)				
0.54		126	0.08 - 2.67	Watanabe et al. (1978)
0.65		20	($\pm$ 0.53)	Buchet et al. (1976)
0.7		16	0.1 - 1.5	Halls and Fell (1981)
Tissues (mg/kg dry weight)[b]				
Liver	7.04	8	($\pm$ 3.11)	Miyata et al. (1983)
	5.8	12	3.6 - 10.4	Lyon et al. (1989)
Brain	1.09[c]	10	0.61 - 3.05	Smeyers-Verbeke et al. (1976)
Kidney:				
cortex	3.87	8	($\pm$ 0.71)	Miyata et al. (1983)
cortex	3.21	36	($\pm$ 1.15)	Pleban et al. (1981)
medulla	2.20	8	($\pm$ 2.20)	Miyata et al. (1983)

TABLE 1 (continued)

Mean concn.		n	Range (or S.D.)	Reference
Heart	1.15	12	0.57 - 1.50	Lyon et al. (1989)
Muscle	0.24	12	0.14 - 0.32	Lyon et al. (1989)

[a] This table is not intended to be an exhaustive list of work of a high standard.
[b] For a more detailed compilation, see Iyengar et al. (1978) or Versieck (1985).
[c] Mean of 11 different brain areas sampled.

concentration of matrix results in spectral and chemical interferences becoming more significant, usually leading to positive errors. In too much of the work in the literature, these factors have been neglected. Versieck and Cornelis (1980) have discussed the variation in reported mean normal concentrations for manganese in serum (range 0.54-34.3 μg/L); they concluded that the consensus mean normal value is 0.5-0.6 μg/L, all other values in the literature being too high because of inadequate care to avoid contamination.

Table 1 summarizes for serum, whole blood, urine and some tissues some measured values for normal subjects which bear further examination as reasonable care was taken in determination.

With these low levels, only the most sensitive analytical techniques are suitable. Two techniques predominate in the literature – neutron activation analysis (NAA) after radiochemical separation and graphite furnace atomic absorption spectrometry (GF-AAS). GF-AAS has a practical detection limit for biological materials of 0.05-0.1 μg/L. Determination of normal values for serum and urine therefore unfortunately requires working rather close to the detection limit. Neutron activation analysis has better sensitivity; it does require a high thermal neutron flux. The sensitivity of the method can be varied by altering the irradiation time and the count time but the limitations will be the size of the blank and the extent of control of contamination. Damsgaard et al. (1973) estimate for their method a detection limit of 0.02 μg/L. The same group (Heydorn et al., 1979), in a study of sources of variability in measurement of manganese in serum by NAA, conclude that the most significant source was contamination during sampling.

Two other techniques which warrant some mention are inductively-coupled plasma atomic emission spectrometry (ICP-AES) and inductively-coupled plasma mass spectrometry (ICP-MS). The former is limited in sensitivity. Bussière et al. (1989) found a detection limit of 2 μg/L which was just sufficient to determine manganese in amniotic fluid directly when matrix-matched calibration and an internal standard of gallium was used. Concentrations in ten samples ranged from 14 to 16 μg/L. Preconcentration techniques using a poly(dithiocarbamate) resin have been applied to the determination of manganese in urine (Barnes et al., 1983; Van Berkel and Maessen, 1988). Barnes et al. (1983) digested the

resin with nitric acid and hydrogen peroxide for direct analysis by ICP-AES with a detection limit of around 0.2 μg/L whereas Van Berkel and Maessen (1988) determined the washed resin directly with graphite furnace vaporization into the plasma to obtain better sensitivity. Results on a urine reference material (RM) agreed with the certified value.

The technique of ICP-MS offers greater sensitivity than ICP-AES but the chances of interference, particularly from isobaric interferences from polyatomic species, are greater. A complete sample digestion helps to remove these. Using a multi-stage digestion with nitric acid and hydrogen peroxide, Friel et al. (1990) obtained results for manganese in NIST Oyster Tissue and Bovine Liver RMs and IAEA Bovine Muscle RM in agreement with the certified values. Their detection limit corresponded to 4 μg/kg in a solid sample. Lyon et al. (1991) similarly demonstrated correct results for manganese on a range of certified tissue RMs, but with a simpler one-stage pressure digestion with nitric acid using microwave heating. Results on human tissue samples taken at autopsy correlated well with results by GFAAS.

Graphite furnace atomic absorption spectrometry

After contamination, the next most difficult problem in determination of manganese by GF-AAS is control of the high background from the matrix at the measurement wavelength of 279.5 nm. Unless Zeeman background correction is used, it is desirable to reduce the background as much as possible to bring the background absorbance well within the working range of the correction system (normally a deuterium arc). Background can be reduced by:
1. Dilution. The use of dilute nitric acid as diluent rather than distilled water reduces the background even further.
2. Use of internal gas flow during atomization.
3. Use of increased time in the ashing step.
4. Use of fast ramp rates in atomization.

Fig. 1 illustrates these points. Negative peaks before the main peak indicate that the correction system is finding difficulty in coping with the background signal.

Dilution is in many cases desirable for other reasons. Serum pipettes easier and dries much more satisfactorily on the surface of the graphite tube or platform when diluted with water. Even better is to dilute with a surfactant (0.25% Triton X-100) which enables the serum to spread more evenly and allows faster drying.

Ashing may be carried out at temperatures up to 1100°C which enables removal of part of the matrix. The matrix that remains can still be a problem. In the author's experience (Halls and Fell, 1981), the effect of the matrix on the signal can be minimized by control of the ashing temperature and time. In this way, simple aqueous standards can be used for calibration. However, the experience of others, particularly in the determination of manganese in serum, was that aqueous calibration could not be used and they used either matrix-matched calibration with a serum low in manganese (Pleban and Pearson, 1979; Subramanian and Meranger, 1985; Nève and Leclerq, 1991) or standard additions on each sample (Casey et al., 1987). Smeyers-Verbeke et al. (1976) have studied the
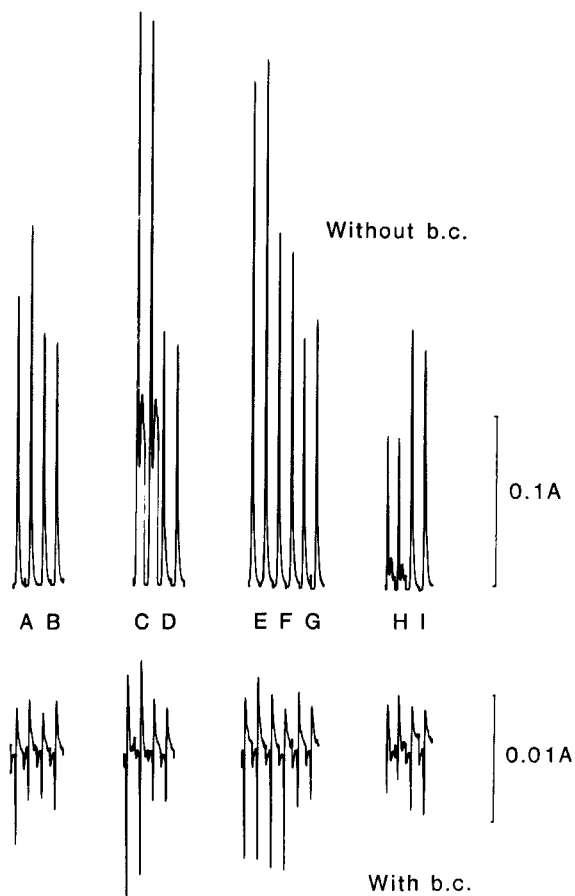
Fig. 1. The effect of various parameters on background absorption in the determination of manganese in urine by GF-AAS. Recorder traces are shown for a normal urine (concentration 0.4 μg/L) diluted by two, with and without background correction under various conditions: diluent in A, distilled water; B, 0.1 M nitric acid; as B, but with an internal flow rate of C, 10 mL/min; D, 30 mL/min; as D, but with ashing times of E, 10 s; F, 20 s; G, 30 s; as G, but with atomization ramp times of H, 0 s (fast ramp heating) and I, 2 s. Unless otherwise stated, conditions are as in Table 2. Scale expansion for trace without background correction, 2x; for corrected signals, 15x.

interferences of inorganic constituents in biological materials on manganese; magnesium and calcium chlorides caused major interferences which they concluded were due principally to losses of manganese in the ashing stage. By control of the ashing parameters, the effects could be reduced. In all graphite furnace procedures, chlorides are best avoided, and so, for digestions, the oxy-acids nitric or sulphuric acid are preferable. The same group demonstrated the lack of matrix interference in the determination of manganese in NIST Kale Powder, NIST fish meal (Smeyers-Verbeke et al., 1975) and human brain tissue (Smeyers-Verbeke et al., 1976) after digestion with sulphuric acid and hydrogen peroxide.

Baruthio et al. (1988) provided a detailed literature survey on determination of manganese in biological samples by GF-AAS comparing sample preparation, calibration and instrument settings.

It should be emphasized that there are important differences between graphite furnaces produced by different manufacturers which affect the choice of indicated temperatures, ramp rates and internal gas flow rates. Even different models from the same manufacturer have small differences which affect the programme to be chosen (Halls, 1984). It is important that potential users of programmes described later should use this only as a basis and verify the appropriate parameters for their instrument.

Neutron Activation Analysis

Activation is based on the reaction

$$^{55}Mn\ (n, \gamma)\ ^{56}Mn$$
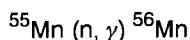
As ^{56}Mn has a half-life of 2.587 h, the determination can only really be carried out in a laboratory very close to a reactor. Some contribution in activity comes from activation of iron:

$$^{56}Fe\ (n, p)\ ^{56}Mn$$
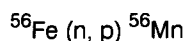
This interfering reaction is principally a problem in the analysis of packed cells which have a high iron concentration. Versieck et al. (1974) overcame this by using a reactor with a high ratio of thermal to fast neutron flux (90) which reduced the interference to 7%; this could easily be corrected for.

After irradiation and cooling, samples are dissolved in acid and carrier added. Separation of manganese can be made by precipitation as tetraphenylarsonium permanganate (Cotzias et al., 1966) or manganese dioxide (Miyata et al., 1983); by extraction at pH 8 into chloroform with 8-hydroxyquinoline (Versieck et al., 1973) or with diethylammonium diethyldithiocarbamate (Damsgaard et al., 1973); by separation with anion exchange resin (Miyata et al., 1973) and after removal of alkali metal salts on a hydrated antimony pentoxide (HAP) column (Miyata et al., 1973). Addition of another manganese isotope (^{54}Mn) can be used to check recoveries of separation procedures.

Sampling

Choice of containers

The choice of containers for collecting samples is important. Containers with caps that have lining inserts or rubber rings for sealing should be avoided. Coloured caps should also be viewed with suspicion as pigments can release a number of trace elements. Polythene bottles and sample tubes of polystyrene or polycarbonate with clear polyethene caps have all proved suitable provided cleaning procedures are carried out first.

Testing the contamination by filling with distilled water and then measuring the manganese concentration will lead to misleading information. Blood and urine are strong electrolytes which will result in aggressive attack on metallic particles; distilled water contains no electrolyte and its attack will be minimal. Testing should be carried out with saline solution, dilute acid or blood or urine itself.

In the author's experience, cleaning containers by filling them with 20% v/v nitric acid, leaving overnight and then rinsing with distilled water has proved satisfactory. Versieck et al. (1973) used high-purity quartz tubes which were cleaned by boiling in a mixture of nitric and sulphuric acids. Damsgaard et al. (1973) used polyethene vials which were cleaned with 3% hydrogen peroxide. Selection of the source of polyethene vials was considered important.

Blood

The contribution that manganese in stainless steel makes to contaminating specimens has been illustrated by Versieck et al, (1982). Needles constructed from other materials have been tried, for example from platinum (Damsgaard et al., 1973). Plastic cannulae are readily available (e.g. Venflon cannulae, Viggo, Sweden) which provide a convenient way of sampling. These consist of a flexible Teflon tube closely surrounding a stainless steel needle which enables the catheter to be inserted in the vein. After insertion, the inner needle is withdrawn leaving in place the plastic catheter, to which a three-way tap and standard disposable syringe are attached. The inner surface of the catheter will still be contaminated by contact with the needle. The first 10 mL of blood taken through the catheter should be discarded or used for less critical tests. The second 10 mL is taken and transferred to a cleaned sample tube. The collection of serum rather than plasma avoids the use of anticoagulants. The use of this technique was effective in reducing a mean normal serum concentration of 1.3 μg/L on samples obtained with ordinary stainless steel needles to a mean of 0.58 μg/L (Halls and Fell, 1981).

In all work on the determination of whole blood concentrations of manganese, stainless steel needles seem to have been used. Since the concentration of manganese in whole blood is at least 10 times greater than in serum, the effect of contamination will be proportionally smaller. Nevertheless if reliable values for manganese in whole blood are to be achieved, the use of special sampling techniques would seem to be necessary.

Urine

Whereas a blood sample is obtained at one time by one person, a 24 h urine collection requires several samplings and, in the case of a patient in hospital, the assistance of several nurses in different shifts. The main problem in avoiding contamination may therefore be in ensuring that the recommended sampling procedure is followed at all times. When this is difficult, the collection of a random sample may be more reliable.

Urine from patients in hospital should be collected in a plastic urine bottle or pan. Alternatively a glass bottle should be used. Stainless steel or disposable fibre bottles or pans should be avoided. The use of disposable fibre bottles, now common in many hospitals, leads to extreme contamination with manganese (Halls and Fell, 1981).

Urine should be transferred to an acid-washed container for storage. Cornelis et al. (1975) found that manganese does not come down with the sediment that forms in urine but remains in solution. No change in concentration was noted on 3 days storage at room temperature. It would seem advisable that if storage for longer periods is anticipated, the urine should be acidified.

Tissues

The use of stainless steel Menghini needles for liver biopsies has been shown to lead to considerable contamination of the specimen, adding up to 45% more manganese (Versieck et al., 1982). When wedge biopsies were taken with stainless steel surgical blades, the contamination was considerably less (< 1%). Generally the amount of contamination would seem to depend on the ratio of surface area in contact with the stainless steel implement to the total volume of specimen. The larger the sample, therefore, the less the contamination. Where stainless steel knives are to be avoided, quartz or titanium knives can be used. Pietra et al. (1990) used stainless-steel knives that had been coated with titanium nitride by a reactive ion-plating technique. The titanium nitride coating had high chemical stability and good wear resistance and was shown to be free of contamination when sampling tissue for determination of cobalt and chromium. Release of manganese from the coated surgical instruments was shown to be minimal in leaching experiments.

PROCEDURES

Serum

Most of the reliable values for manganese in serum have been obtained by neutron activation analysis (Cotzias et al., 1966; Versieck et al., 1974; Damsgaard et al., 1973). Graphite furnace atomic absorption spectrometry can achieve a faster rate of analysis but it is important that adequate care be taken in sampling, handling and analysis to achieve the degree of accuracy found in the best work by neutron activation. Too many of the results by GF-AAS are too high. The actual determination is relatively straightforward.

Matrix effects are minimal provided sufficient ashing at 1100 °C is used. As indicated previously, background is a problem and Zeeman systems are preferable (Pleban and Pearson, 1979; Nève and Leclerq, 1991). With deuterium-arc background correction, greater attention has to be paid to the design of the programme to reduce background.

It is apparent from two reports (Subramanian and Meranger, 1985; Nève and Leclerq, 1991) that platform atomization offers no advantage for this determination. A method using platform atomization has been described (Paschal and Bailey, 1987) but no comparison was made with wall atomization.

As indicated previously, some dilution of the sample is normally necessary, normally with a dilute solution of a surfactant such as Triton X-100 (Nève and Leclerq, 1991; Paschal and Bailey, 1987; Pleban and Pearson, 1979). Favier et al. (1982) found that 10% v/v ethylene glycol was a suitable diluent, but 5-fold dilution was necessary to obtain satisfactory precision with an injection of 50 μL. However, maximum sensitivity is needed and if should not be necessary to dilute more than 2-fold. For Subramanian and Meranger
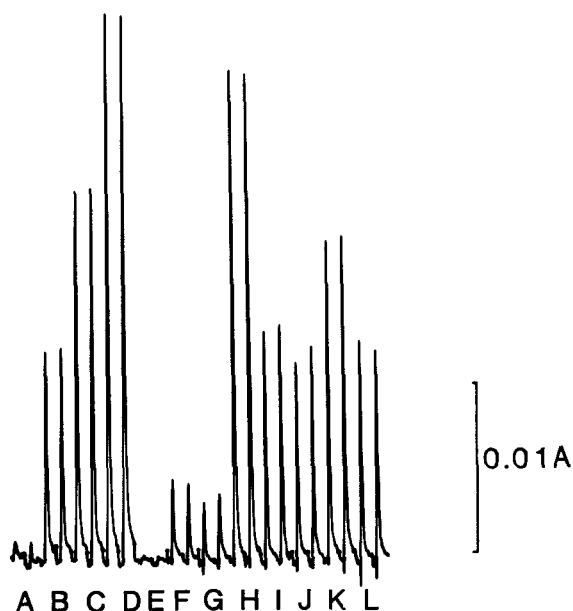
Fig. 2. Recorder trace for the determination of manganese in serum by GF-AAS. Method as described in the text. Duplicate injections of A-D, standards of 0, 1, 2 and 3 μg/L respectively; E, blank; F and G, sera from normal individuals; H-L, sera from patients on total parenteral nutrition at home. Sera are diluted by 2 as in the method.

(1985), however, simple dilution of serum, even with a solution of Triton X-100, resulted in a carbonaceous residue in the graphite tube. To overcome this, samples were deproteinized with an equal volume of 5% v/v nitric acid and the supernatant analyzed using calibration with matrix-matched standards. Formation of carbonaceous residues from serum may be a problem with pyrolytic graphite tubes but others (Nève and Leclerq, 1991; Casey et al., 1987; Favier et al., 1982) seem to have satisfactorily overcome the problem. Uncoated tubes have a porous structure which enables the sample to spread more evenly and with these, in the author's experience, this problem has never been found.

The method below is based on a previously described procedure (Halls and Fell, 1981). Here dilution, some gas flow during atomization and an ashing time of 30 s are used to reduce background. Unfortunately, the introduction of fast ramp heating in the atomization stage with this programme resulted in a matrix affect, which was not apparent when a 2 s ramp time was used. A 2 s ramp time is therefore used in this programme. To enable spreading of the serum on the graphite tube to assist drying, dilution with 0.25% Triton X-100 is used. Fig. 2 shows results obtained with this method.

Procedure

Samples and standards (0, 2, 4, and 6 μg/L manganese in 0.1 mol/L nitric acid are diluted 1 + 1 with a diluent of 0.25% v/v Triton X-100 in 0.001 mol/L nitric acid. These are then analyzed using the conditions in Table 2.

TABLE 2

INSTRUMENTAL CONDITIONS FOR THE DETERMINATION OF MANGANESE BY GRAPHITE FURNACE ATOMIC ABSORPTION SPECTROMETRY

Wavelength	279.5 nm	Injection volume	20 μL
Slit Width	0.7 nm	Background correction	ON
Scale expansion	10 x	Uncoated graphite tube	

FURNACE PROGRAMMES

Designed for the Perkin-Elmer HGA 500 furnace. Instrument dependent.

Serum				Urine			
Stage	Temp (°C)	Ramp Time (s)	Hold Time (s)	Stage	Temp (°C)	Ramp Time (s)	Hold Time (s)
1. Dry	120	1	15	1. Dry	140	1	7
2. Ash	1100	2	30	2. Ash	1100	2	30
3. Atomize	2700	2	8*	3. Atomize	2600	0	8*
4. Clean	2700	1	5	4. Clean	2700	1	5

* Autozero at - 2s, Record at -1s, Internal flow 30 mL/min.

Whole blood

Whole blood has been suggested as a good indicator of body manganese (Keen et al. 1983). In analysis, the minimum of pretreatment is preferable. Methods have been described for GF-AAS which involve extraction after digestion of the blood (Buchet et al., 1976). However, direct determination was shown to be possible by Muzzarelli and Rocchetti (1975) who diluted blood 1 + 5 with distilled water and calibrated by standard additions. A similar approach was used by Pleban and Pearson (1979) who diluted blood 1 + 3 with 1 g/L Triton X-100 solution. Matrix-matched standards were prepared from a whole blood sample. Zeeman-effect background correction was used in their method, but it is not essential to use a Zeeman system. Most deuterium-arc background correction systems should be able to cope with the background. Tsalev et al. (1977) even found that, with an injection of 2 μL of whole blood in a Varian-Techtron miniature furnace, analysis could be made without background correction. It is unlikely that this applies to all furnaces. Oxygen ashing within the furnace programme was found by Hams and Fabri (1988) to be beneficial in reducing background absorption. Samples were diluted 1 + 7 with a dilute solution of Triton X-100 and concentrated nitric acid was injected onto the platform after the sample. Although platform atomization was used and was claimed to be the reason why simple aqueous calibration was possible, no comparison with wall atomization was recorded.

Allain et al. (1987) issued a cautionary note about attempting to use acid precipitation in the measurement of manganese in whole blood. Low results were obtained which they suggested was because the manganese, bound to haem in erythrocytes, was precipitated with nitric acid and not released into solution.

The procedure below is based on that of Pleban and Pearson (1979).

Procedure
Prepare standards of 0, 2, 5 and 10 μg/L manganese in 0.1% v/v Triton X-100 solution. Dilute a blood sample low in manganese 1 + 3 with the standards to prepare calibration solutions equivalent to 0, 6, 15 and 30 μg/L manganese in the blood samples. Samples are diluted 1 + 3 with the zero standard. A blank of distilled water diluted similarly is also prepared. 10 μL aliquots of prepared standards, blank and samples are analysed in duplicate in the furnace using the programme for serum shown in Table 2. Subtract the peak absorbance of the standards from the value for the zero standard and plot a calibration curve. From the peak absorbances of the samples, subtract the value for the reagent blank and read the concentration from the calibration curve.

Urine

Determination of manganese in urine can be made by flame AAS after extraction into an organic solvent (Van Ormer and Purdy 1973) and after ion-exchange separation (Pantucek, 1981). Solvent extraction procedures have also been described for GF-AAS (Buchet et al., 1976; Watanabe et al., 1978). As the direct determination of manganese in

urine by GF-AAS has been demonstrated to be straightforward (Halls and Fell, 1981), solvent extraction procedures should no longer be necessary. Frech et al. (1985) have shown that the related technique, electrothermal atomic emission spectrometry, gives better detection limits. Practically the determination is similar to GF-AAS and it also allows direct calibration against aqueous standards.

In direct determination, the main problem, as with serum, is to reduce background absorption; matrix interferences are minimized by using an ashing stage of 1100 °C for at least 20 s. The procedure below is based on that described previously (Halls and Fell, 1981) and uses the same techniques to reduce background as for serum determination. Here, however, fast ramp heating may be used to further reduce background; no matrix effect is introduced in this case. The drying stage has been reduced to a time of 8 s as was previously found satisfactory for the determination of urine copper (Halls, 1984).

Procedure

Urine samples are diluted 1 + 1 with 0.1 mol/L nitric acid and analysed against standards of 0, 2, 4 and 6 μg/L manganese in 0.1 mol/L nitric acid using the conditions shown in Table 2.

Tissues

Neutron activation analysis has the advantage that virtually no pretreatment of tissue samples is necessary before irradiation. Operations after irradiation will not be affected by the problem of contamination since only the radioactive isotopic form of manganese is measured. Thus many studies of tissues have used this technique (for example, Cotzias et al., 1968; Miyata et al., 1983).

For GF-AAS, samples are normally first brought into solution by wet digestion. Sulphuric acid with hydrogen peroxide (Smeyers-Verbeke et al., 1976), mixtures of nitric acid and sulphuric acids (Belling and Jones, 1975) and nitric acid alone (Bonilla, 1978) have been used. Belling and Jones (1975) used a subsequent extraction of the manganese with cupferron into methyl isobutyl ketone to avoid matrix interferences. Extraction is not essential, as was shown by Bonilla (1978) who used a standard additions calibration on the nitric acid digest. The approach of Smeyers-Verbeke et al. (1976) is even more straightforward in that, with sufficient dilution of the digest, analysis may be made directly against standards in 4% v/v sulphuric acid.

An alternative approach in GF-AAS is direct sampling of a slurry of finely-divided powder of the dried tissue material. Jordan et al. (1989) showed that this could be a rapid and accurate technique for determination of manganese. Slurries of 10 mg of sample in 1 mL of 5% v/v nitric acid, 0.04% Triton X-100 were maintained in suspension by ultrasonic agitation of the autosampler cups. Measurement was made at the less-sensitive 403.1 nm line and, by omitting the ashing stage and adding no modifier, the cycle time was kept down to about two minutes. Reliability was demonstrated by analysis of a number of biological reference materials, including NIST SRM Bovine Liver.

The procedure below uses the simple digestion with nitric acid described by Bonilla, but avoids trying to determine the digest directly. Concentrated nitric acid attacks graphite tubes quite badly and can lead to premature fracture. Digests are diluted to an equivalent concentration of 10% v/v nitric acid and analysed against standards in the same matrix.

Procedure

Digest approximately 100 mg of dry tissue with 2 mL nitric acid in an acid-washed borosilicate glass test tube in a heating block. When no more brown fumes are evolved, allow to cool and make up to 25 mL. Analyse against standards of 0, 5, 10 and 15 μg/L manganese in 10% nitric acid using the programme for urine in Table 2. This effectively covers the range 0-3 mg/kg. For other sample weights, adjust the volume of acid and dilution accordingly.

SPECIATION

The difficulties of measuring total manganese in biological fluids indicate that, with the present state of technology, studies of speciation in biological fluids are very difficult and likely to remain a considerable challenge in future years. Tracer studies with the isotope ^{54}Mn, however, allowed an evaluation of the movement of manganese through the body (Cotzias and Greenough, 1958; Borg and Cotzias, 1958) and have shown that manganese in serum is located on the β_1-globulins. It is suggested that manganese is bound, probably as Mn(III), to a specific globulin, transmanganin (Cotzias and Bertenchamps, 1960). In erythrocytes, manganese is believed to be incorporated as a manganese porphyrin (Borg and Cotzias, 1958). Milne et al. (1990) measured the manganese content of platelets, mononucleated cells, polynucleated cells and erythrocytes and found that about 66% of the manganese in blood was associated with the erythrocytes and about 30% with the platelets and leucocytes. Separation was made on a discontinuous gradient of colloidal polyvinylpyrrolidone-coated silica (Percoll). The cells were digested with nitric acid and hydrogen peroxide for determination of manganese by GF-AAS.

Much is known about the enzyme functions of manganese in living organisms from studies in animals and bacteria and about the co-ordination chemistry of manganese with ligands found in biological systems. These aspects have been reviewed by Keen et al. (1984) and Sawyer (1978).

REFERENCE MATERIALS

This subject will be dealt with elsewhere in this book, but it is worth noting the difference this has made to the history of the determination of manganese. Throughout the 70s and early 80s, the only RMs available with certified or recommended values for manganese were tissues, such as the Bovine Liver SRM from NBS (now NIST). For assessing bias at the low concentrations found in serum, this was not at all suitable. More recently, Veillon et al. (1985) produced a serum (NIST RM 8419) with a recommended

manganese concentration of 2.6 ± 0.5 μg/L. This was a step forward, but the concentration was still outside the normal range. A major contribution was made by Versieck et al. (1988) who produced a freeze-dried human serum with dedicated care and attention to ensure that the natural concentrations were minimally affected by processing. This "second-generation" RM was certified for manganese by NAA and GF-AAS (7.7 ± 0.3 μg/kg dry weight or 0.70 ± 0.3 μg/L after reconstitution). The availability of this material was of great assistance in the optimization of a recent method for determination of manganese in serum by GF-AAS (Nève and Leclerq, 1991). Progress still needs to be made as the Versieck material reconstitutes to a turbid material with a viscosity greater than that of normal human serum. This may therefore give problems of reproducibility with some methods which behave quite satisfactorily with normal samples.

CONCLUSIONS

The problems in the determination of manganese in biological material have been highlighted in many papers in the literature, as reviewed in this chapter. Despite this, papers still appear with data that is of little value because the proper precautions in sampling and determination were not taken. There are emerging now reliable values for normal levels of manganese in body fluids and tissues; new studies should show control values which are similar to these.

Further progress can certainly be made in analysis, particularly in increasing sensitivity. However, the most important questions regarding manganese remain in the use of these determinations – what is the most reliable material to use for evaluation of manganese status (serum, whole blood, hair or nails) and is manganese really essential for humans?

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Mercury

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INTRODUCTION

Mercury (Hg) occurs in nature as a mixture of seven stable isotopes; the average atomic mass of the blend is 200.6. The atomic masses and natural abundances of the isotopes are: 196.0 (0.15 %), 198.0 (10.1 %), 199.0 (17 %), 200.0 (23.1 %), 201.0 (13.2 %), 202.0 (29.65 %) and 204.0 (6.8 %). Two radioactive isotopes, ^{197}Hg and ^{203}Hg, have been widely used in toxicological studies, radiometric analysis, and also in checks of yield of analytical procedures. The fact that mercury is an isotope mixture may be of importance in analytical work using mass spectrometry, since there are no reference samples with well-defined isotope composition.

Mercury may occur in three oxidation states: 1) Elemental or metallic mercury, Hg(0), 2) monovalent mercury, Hg(I), and 3) divalent mercury, Hg(II).

At room temperature, elemental mercury is a liquid; it has a considerable vapour pressure. Metallic mercury has a rather low solubility in water and lipids. In the presence of oxygen, metallic mercury is slowly oxidized into Hg(II). Oxidation occurs also in the body (cf. Clarkson et al., 1988a).

Hg(I) salts contain the dimer ion Hg_2^{2+}. They are difficultly soluble in water; the nitrate, chlorate, and perchlorate excepted. Dissolved Hg(I) salts dissociate readily into hydrated Hg_2^{2+}. Among Hg(II) salts, the chloride, cyanide, sulfate, and nitrate, are water soluble; others, like iodide, sulfide and selenide, are insoluble. In solutions, Hg(II) halides and Hg(II) cyanide are poorly protolyzed; the Hg(II) ion forms strong complexes with the anions. Hg(II) ions easily bind to sulfhydryl groups in proteins.

Mercury(II) may bind covalently to carbon in a great number of organo-metallic compounds, of the types RHg+ and RHgR', where R and R' are the organic moieties.

Of the organomercurials, the short-chain alkylmercury(II) compounds form salts with the halogens, which are highly volatile, while hydroxides, nitrates, and in particular dicyandiamides, are less volatile. The short-chain alkylmercurials are highly soluble in organic solvents and lipids. Further, they easily bind mainly to sulfhydryl groups, and thus mainly exist in protein-bound form in biological material.

ENVIRONMENTAL AND TOXICOLOGICAL SIGNIFICANCE

There are several reviews on different aspects of mercury toxicology (Friberg and Vostal, 1972; WHO, 1976, 1980, 1989a, 1989b, 1990, 1991; Skerfving and Berlin, 1985; Berlin, 1986; Clarkson et al., 1988a).

Mercury is ubiquitously present in the environment. Some of the mercury originates from natural sources, some is the result of pollution. A detailed discussion of environmental aspects of mercury can be found in a recent review (WHO, 1989a). Here only aspects relevant to human exposure will be briefly reviewed.

Human exposure

Fish and marine mammals and birds preying on fish may contain considerable amounts of mercury (WHO, 1989b). The mercury in fish originates from mercury in water, which is methylated, and accumulates in the fish as such. Fish in "non-polluted" water areas may contain 0.01-0.05 mg/kg; however, fish of prey has often higher levels. Fish in heavily mercury-contaminated waters may have up to 20 mg/kg.

The average mercury intake through Swedish *food* is in the order of 10 μg/day (Dencker and Schütz, 1971; Schütz, 1979). Fish plays a major role (Jonsson et al., 1972). Subjects who have a high fish intake may thus ingest large amounts of methylmercury, 1 mg/day, or even more (Swedish Expert Group on Methylmercury in Fish, 1971; Birke et al., 1972; Skerfving, 1974). The maximum tolerable weekly intake is 0.3 mg of total mercury and 0.2 mg of methylmercury (WHO, 1989a).

An important source of exposure to inorganic mercury in man is *amalgam* dental fillings (cf. Swedish Expert Group on Mercury in Amalgam, 1987; cf. Clarkson et al., 1988b; Nylander et al., 1989; Molin et al., 1990a and 1991; Åkesson et al., 1991). Mercury vapour is slowly released from the surface of the fillings. The absorbed amount has been estimated to be about 3-18 μg/day by use of data from different studies (cf. Clarkson et al., 1988b).

Mercury and its compounds earlier had a wide use in *drugs*. Nowadays, the use is limited, and then mainly as organomercurials (thiomersal, mercurochrom, and phenylmercury salts) in disinfectants and preservatives.

There is exposure to mercury in several types of *occupational* settings. Most of the occupational exposure is to inorganic mercury, mainly elemental mercury vapour. Among work operations causing such exposure is mercury mining and work in instrument, fluorescent tube, and chloralkali factories (Barregård et al., 1987; Erfurt et al., 1990). In the latter case, there is also some exposure to inorganic mercury salts. Further, the produc-

tion of alkaline batteries with amalgamated zinc electrodes may involve high exposure to mercury (Roels et al., 1991). A widespread, but low-intensity, exposure to elemental mercury vapour is present in dental surgeries (Herber et al., 1988; Åkesson et al., 1991). Spill of mercury in poorly ventilated chemical and physical laboratories may still constitute a health risk, if not properly collected.

In addition, some occupational exposure to organic mercury compounds may occur in some countries, where alkyl- (mainly methyl-) and alkoxy- (mainly methoxyethyl-) mercury salts are used as seed dressings (Skerfving and Copplestone, 1976), and phenylmercury is sometimes used as a fungicide.

Metabolism and toxic effects

Different chemical forms of mercury have quite different metabolism and toxic effects. Thus, analytical specification of the mercury may be crucial. As most of the exposure to mercury in man is to metallic mercury and methylmercury, the comments here will largely be restricted to these two compounds.

Inorganic mercury

Elemental mercury vapour is efficiently absorbed from the respiratory tract (Nordberg and Skerfving, 1972; Einarsson et al., 1974; Skerfving and Berlin, 1985; WHO, 1991; Berlin, 1986; Clarkson et al., 1988a). Metallic mercury is poorly absorbed from the gastro-intestinal tract and through the skin. After absorption, mercury vapour is dissolved in the blood and remains as such for a short time. In the blood, the levels of mercury are similar in blood cells and plasma (Einarsson et al., 1974; Barregård et al., 1992). The mercury may pass the blood-brain barrier into the nervous system. A major part of the body burden is in the kidney. Also, mercury passes the placenta barrier. In the blood and in the tissues, the elemental mercury is oxidized into Hg(II) in a reaction catalyzed by the enzyme catalase. Mercury is mainly excreted in the urine; elimination occurs also through the fecal route, to some extent as metallic mercury (Henderson et al., 1974). In addition, there is some exhalation of Hg(0) vapour. Inorganic mercury also occurs in milk (Skerfving, 1988),

Exposure to elemental mercury vapour may cause effects on the central nervous system, with a change of personality and tremor (cf. Skerfving and Vostal, 1972). Also, mercury may affect the kidney; this may occur as a tubular (Barregård et al., 1987) and/or glomerular (cf. Berlin, 1986; WHO, 1991) malfunction. Further, mercury may provoke hypersensitivity with skin manifestations.

Mercury from Hg(II) salts is absorbed only to a limited extent from the gastro-intestinal tract (cf. Berlin, 1986; WHO, 1991). The Hg(II) accumulates mainly in the kidney; it passes the blood-brain barrier only to a limited extent. Mercury(I) is unstable in biological material; in the presence of sulfhydryl groups it undergoes disproportionation into one atom of metallic mercury and one ion of Hg(II).

There is an interaction between inorganic mercury and selenium, which affects both mercury metabolism and, in animal experiments, toxic effects (cf. Skerfving, 1978). In tissues, a considerable fraction of inorganic mercury may be co-deposited with selenium (Kosta et al., 1975; Magos et al., 1984; Yoshinaga et al., 1990; Nylander and Weiner, 1991). The association seems to be strongest in the kidney (Rossi et al., 1976), while less convincing for brain (Rossi et al., 1976; Cappon and Smith, 1981; Ehmann et al., 1987).

Organic mercury

Methylmercury (MeHg) has metabolic and toxic effects which are significantly different from those of metallic mercury. It is efficiently absorbed from the gastro-intestinal tract (Clarkson et al., 1988a; WHO, 1990). The absorption through intact skin is probably also significant. In the blood, MeHg is mainly present in the cells (Birke et al., 1972; Skerfving, 1974). It passes the blood-brain and placenta barriers (cf. Clarkson et al., 1988a; WHO, 1990). Within the brain, some of the MeHg is decomposed into Hg(II) (cf. WHO, 1990). Methylmercury is mainly excreted *via* the feces. Elimination through milk also occurs (Skerfving, 1988).

Mass poisoning by MeHg in man has occurred through intake of contaminated fish (cf. Swedish Expert Group on Methylmercury in Fish, 1971) and dressed seed (Skerfving and Copplestone, 1976). In the adult subject, MeHg causes central nervous system disease, with paresthesia, ataxia, and concentric restriction of the visual fields (cf. Skerfving and Vostal, 1972). Methylmercury is genotoxic in man (Skerfving et al., 1972). In the fetus, MeHg may cause severe central nervous system disturbances (cf. Clarkson et al., 1988a; WHO, 1990). Probably, fetal MeHg exposure at lower levels causes disturbances of the mental development of the infant (Kjellström et al., 1989).

In animal experiments, there is a metabolic interaction between MeHg and selenium, which, to some extent, changes the toxicity of MeHg (cf. Skerfving, 1978; WHO, 1990).

Other organomercurials. Alkylmercury compounds other than MeHg are not found in natural biological samples. Compounds like phenylmercury and alkoxyalkylmercury rapidly decompose in the body into Hg(II), which binds to different ligands (cf. Skerfving, 1972a).

Levels in tissues and excreta

Blood

Blood is often used for biological monitoring of exposure to Hg(0) vapour and MeHg. The type of compound is determinative for the distribution of mercury between blood cells and plasma.

In *blood cells*, the level is greately influenced by fish intake (MeHg). In non-fish eaters, a survey of 98 international publications, mainly from the 1970's and 80's, indicated an average of 3.8 μg/L (Brune et al., 1991). In recent Swedish studies, the level was about 2 μg/L (Svensson et al., 1987 and 1992). In subjects with an extremely large intake of

mercury-contaminated fish, the level may even exceed 1,000 μg/L (Birke et al., 1972; Skerfving, 1974). In persons without particular mercury intake, a minor fraction of the mercury in blood cells is MeHg (cf. Skerfving and Berlin, 1985), while in subjects with a high fish intake, this compound is dominating (cf. Clarkson et al., 1988a).

The mercury level in *blood plasma* is sometimes used for monitoring of exposure to inorganic mercury. The plasma level is affected comparatively more than the whole blood level by mercury exposure from amalgam. In subjects without amalgam fillings, the average level was about 1 μg/L (Molin et al., 1990b and 1991; Åkesson et al., 1991), and in subjects with many amalgam fillings about 3 μg/L (Åkesson et al., 1991). In subjects with occupational exposure, the level in plasma may be further increased, ten times or more (Erfurth et al., 1990; Langworth et al., 1991).

Whole blood may contain 1-2 μg/L in subjects without amalgam fillings and with low fish consumption (Svensson et al., 1987 and 1992; Molin et al., 1990a and 1991; Åkesson et al., 1991). In persons with occupational exposure, levels ranging from 20-100 μg/L are common (Erfurth et al., 1990; Langworth et al., 1991; Barregård et al., 1992), and in subjects with heavy consumption of contaminated fish, levels above 500 μg/L have been observed (Birke et al., 1972; Skerfving, 1974).

Other tissues

In the *brain* of subjects without occupational mercury exposure, the lowest reported levels are about 10 μg/kg (Ehmann et al., 1986; Nylander et al. 1987 and 1989; Thompson et al., 1988; Nylander and Weiner, 1991). However, five to ten times (cf. Skerfving, 1972b; Mottet and Body, 1974; Cappon and Smith, 1981; Matsuo et al., 1989; Yoshinaga et al., 1990), or even higher (Yukawa et al., 1980; cf. Hargreaves et al., 1988) total mercury levels have also been reported. Such levels may be explained by a high exposure to mercury through fish diet or a high dental amalgam load, but also analytical problems may have played a role. At the lowest brain mercury levels, probably inorganic mercury constitutes the major fraction (Nylander et al., 1987), while at higher levels, the dominating fraction seems to be MeHg (Matsuo et al., 1989; Yoshinaga et al., 1990).

In subjects with intense and enduring occupational exposure to metallic mercury vapour, or with a high consumption of fish from heavily mercury-polluted waters, the levels in brain may attain the mg/kg level (cf. Swedish Expert group on Methylmercury in Fish, 1971; Berlin, 1986; Clarkson et al., 1988a; WHO, 1990). Considerably elevated mercury levels in brain have been found in occupationally exposed or intoxicated subjects still many years after end of exposure (Kosta et al., 1975; Hargreaves et al., 1988; Nylander and Weiner, 1991).

The mercury level in *cerebrospinal fluid* in subjects without particular exposure is extremely low, in the order of 0.01 μg/L (cf. Swedish Expert Group on Mercury in Amalgam, 1987); it is not clear what the levels are in subjects with particular exposure to mercury.

The mercury level in *kidney* in subjects without particular exposure has been reported to be in the range 20-800 μg/kg (Skerfving 1972b; Mottet and Body, 1974; Nylander et al.,

1987; Nylander and Weiner, 1991). The level seems to be dependent upon the number of amalgam fillings (Nylander et al., 1987), but also geographical differences may be of importance (Stein et al., 1974). The levels in subjects with mercury poisoning have been two orders of magnitude higher (cf. Skerfving 1972b; Berlin, 1986; Clarkson et al., 1988a; WHO, 1991).

Excreta

The mercury level in *hair* is, to a considerable degree, affected by the intake of MeHg. The average level in subjects with a low seafood intake is less than 1 mg/kg (Skerfving, 1972b; Ryabukhin, 1980). It may increase by 1-2 orders of magnitude in persons with a high intake of contaminated fish (Birke et al., 1972; Skerfving, 1974; cf. Clarkson et al., 1988a; WHO 1990) or whale meat (Grandjean et al., 1992). The mercury in hair is mainly present as MeHg (cf. Clarkson et al., 1988a).

Urine is used for monitoring of exposure to inorganic mercury. Correction of variations in dilution by creatinine, density, or osmolality greatly reduces the variation in mercury concentration between spot samples (Barber and Wallis, 1986). The average mercury levels in urine in subjects without particular exposure, and without dental amalgam fillings, is about 1 µg/L (about 1 µg/g creatinine; Molin et al., 1990b and 1991; Åkesson et al., 1991). In subjects with many amalgam fillings, the level is higher, about 5 µg/L (Åkesson et al., 1991). In workers with occupational exposure to Hg(0) vapour, the level may be 2-3 orders of magnitude higher (cf. Berlin, 1986; Barregård et al., 1987; Clarkson et al., 1988a; Erfurth et al., 1990; Langworth et al., 1991; Roels et al., 1991; WHO, 1991). After a brief exposure to Hg(0) vapour, the peak level in urine occurs after several days/weeks (Molin et al., 1990a; Barregård et al., 1992).

The level in *human milk* seems to be about the same as in the plasma of the woman (Skerfving, 1988). Significant regional differences have been reported (Parr et al., 1991).

ANALYTICAL CONSIDERATIONS

Combinations of a broad spectrum of sample-preparation procedures, with many, quite different quantification techniques, have, during the last decades, resulted in thousands of publications on mercury determination in biological materials. Among previous reviews on this matter may be mentioned Manning (1970a; atomic absorption), Lindstedt and Skerfving (1972), Smith (1972), Uthe and Armstrong (1974), Chilov (1975), Ure (1975; atomic absorption and fluorescence), Zmijevska (1977; activation analysis), Nitschke et al. (1989) and Shimomura (1989).

On the way towards final, reliable analytical results, the following steps, each of them equally important, must be considered: (1) Sample collection, (2) sample storage, (3) sample preparation, and (4) mercury quantification. This review is focused on different mercury quantitation techniques, but also the other steps will be discussed in some detail.

Sampling and sample storage

General aspects

Sampling and sample handling are important aspects of any determination of mercury in biological specimens. Mercury is present almost everywhere. Thus, at all sampling for mercury determination, great care must be taken not to contaminate the samples. The risk is, of course, greatest in sampling of mercury exposed workers at their work place. Blood samples should be obtained only after careful cleaning of the skin. Urine samples should only be voided after the worker has taken a shower and changed clothes. In addition, intermediate sample handling, such as withdrawal of sub-samples, must be avoided in e.g. medical consulting offices and laboratories, which may be contaminated with mercury from broken thermometers and other equipment.

The sampling and storage containers, and any reagents used for sample preservation, have to be checked for mercury contamination at an appropriate level. Special cleaning procedures, such as washing with hydrochloric or nitric acid, might be necessary. However, storage of samples does not only involve a risk of *contamination*; also the possibility of *loss* of mercury has to be considered (see Aqueous samples section).

When, for some reason, samples have to be dried before storage, lyophilization should be preferred to drying at elevated temperature (see Standard solutions section).

In biological material, inorganic as well as organic mercury is often strongly bound to the sample matrix, and thus stabilized. Decomposition of MeHg was found to be insignificant in hair, and in fresh and dried fish muscle during long (years) storage (Horvat and Byrne, 1992), while the same study indicated loss from repeatedly deep frozen and unfrozen shellfish and blood. Our own experience indicates stable levels of both organic and inorganic mercury in erythrocytes stored for 1.5 y in refrigerator. Loss of ethylmercury from experimental rat tissue samples, stored at room temperature for one day, has been reported (Brooks et al., 1986).

Aqeous samples

The storage of aqueous samples containing low levels (μg/L) of mercury may result in considerable loss of the analyte (Rosain and Wai, 1973; Feldman, 1974; El-Awady et al., 1976; Riisgård and Hansen, 1990). The reason for this is that mercury, probably after reduction by bacteria (Magos et al., 1964; Avotins and Jenne, 1975), or organic substances (Toribara et al., 1970), diffuses through polymer materials (Avotins and Jenne, 1975; Heydorn and Damsgaard, 1982; Krivan and Haas, 1988; Shimomura, 1989). In this connection, reduction of Hg(II) to Hg(I), followed by disproportionation into Hg(0) and Hg(II), may be of importance (Toribara et al., 1970; Baltisberger et al., 1979; Pinstock and Umland, 1985; Shimomura, 1989). Further, mercury ions and compounds adsorb to surfaces of glass and polymer materials, as demonstrated by use of radiotracer techniques (Greenwood and Clarkson, 1970; Litman et al., 1975; Dogan and Haerdi, 1978;

Stuart 1978b). A significant fraction of mercury adsorbed onto glass was not removed even after several washings with concentrated nitric acid (Litman et al., 1975).

To avoid loss of mercury from dilute aqueous solutions (e.g. natural water samples and dilute working standards) by adsorption or by volatilization, possibly after reduction to Hg(0), different preservatives have been proposed, e.g. cysteine (LaFleur, 1973; Weiss and Shipman, 1976), gold(III) in dilute nitric acid (Christmann and Ingle, 1976; Dogan and Haerdi, 1978), hydrochloric acid (Stoeppler and Matthes, 1978; Louie, 1983; Adeloju and Mann, 1987), hydrochloric acid with hydrogen peroxide (Krivan and Haas, 1988), nitric or sulphuric acid (Omang, 1971), sodium chloride-Na_2EDTA-cysteine (Magos and Cernik, 1969), nitric acid with potassium dichromate (Feldman, 1974; El-Awady et al., 1976; Christmann and Ingle, 1976), nitric acid with potassium permanganate (Piccolino, 1983), potassium permanganate (Avotins and Jenne, 1975), potassium persulfate (Trujillo et al., 1974), and sodium chloride (Weiss et al., 1976; Knechtel, 1980). The stabilizing effect of cysteine may be attributed to the stable complexes with MeHg, and, even more, with Hg(II) (Stary and Kratzer, 1988).

Weiss et al. (1976) demonstrated that loss of mercury from spiked (20-65 ng/L) samples of natural waters stored in polyethene (linear) bottles could be prevented, to a large extent, merely by pretreatment of the bottles with hot, nitric acid. Also, pretreatment of polyolefine bottles with chloroform and aqua regia vapour reduced the loss of mercury substantially (Heiden and Aikens, 1979). This treatment was found superior to the addition of nitric acid with potassium dichromate. The data on the preserving capacity of the different additives is, however, sometimes contradictory. E.g. poor results were reported for nitric acid (Litman et al. 1975), and nitric acid with potassium permanganate (Feldman, 1974; Christmann and Ingle, 1976). Hamlin (1989) presented a review (39 references) on preservation of water samples for mercury analysis, and spoke in favour of nitric acid with potassium dichromate. When comparing the adsorption of Hg(II) onto polytetrafluoroethene (PTFE), glass and polyethene, Litman et al. (1975) found the lowest adsorption to PTFE and the highest to polyethene.

Aqueous alkylmercury samples should be protected from daylight, since they decompose at UV irradiation (Ahmed and Stoeppler, 1986). Olson (1977) found, by use of radioactively labelled MeHg ([203]Hg and ^{14}C), that mercury was lost to a higher degree (up to 20 % in 4 days) from NaCl-solutions (0.5 M) and artificial seawater than from deionised water and from acidic or alkaline solutions. It should be emphasized that the addition of preservatives does not preserve the specificity of the mercury compounds. Thus, considerable decomposition of MeHg has been reported by use of cysteine (Clarkson and Greenwood, 1970) dilute nitric acid (Stoeppler and Matthes, 1978; Oda and Ingle, 1981), potassium dichromate (Oda and Ingle, 1981), and hydrochloric acid (2.2 M) with sodium chloride, or dilute acids with hydrogen peroxide (Ahmed and Stoeppler, 1986).

Contamination of aqueous solutions by diffusion of mercury vapour from the ambient air into plastic containers has also been reported (Bothner and Robertson, 1975; Cragin, 1979; Heydorn and Damsgaard, 1982). The rate of contamination was considerably increased (several μg per month per Liter) when the solutions contained oxidizing preservatives, such as nitric acid or potassium permanganate.

Blood

Blood samples are best obtained in metal-free evacuated tubes containing heparin (without mercury as a preservative). Cells and plasma may be separated in order to determine the mercury distribution between plasma and blood cells and, thus, get a picture of which type of mercury compound the subject has been exposed to. The samples may be stored for a couple of days before significant hemolysis occurs. The samples may be refrigerated at 4°C or frozen until analysis. As the levels in cells and plasma may differ, it is essential to shake whole blood samples before analysis.

In our experience, the mercury level in blood samples, collected in evacuated tubes, is stable at least one month when stored at room temperature, and for years when refrigerated. However, it is possible that a slight, initial loss may occur at handling of blood samples from subjects who have immediately before been exposed to high levels of Hg(0) vapour (Barregård et al., 1992).

Other organs

Other organs may be sampled during surgery or at autopsy. In some organs, the mercury levels in subjects without particular exposure is so low, that great care to avoid contamination must be exercised, e.g. by use of knives made from quartz or titanium.

In fish, mercury is usually determined in the muscle, where it has a fairly even distribution.

Excreta

Hair samples are obtained by clipping. If assessment of recent exposure is the prime object, hair close to the scalp should be sampled. If earlier exposure is of interest, strands of hair are sampled, and care is taken not to disturb the relationship between the hairs, e.g. by taping them together. The hair may then be cut into pieces; a length of one cm corresponds to about one month. If the objective is to measure mercury incorporated into the hair, when it was formed, washing of the hair with detergents may be done. However, although several procedures have been proposed, there is no satisfactory washing method.

Urine samples may be sampled in acid-washed plastic or glass containers. There is a diurnal variation of mercury excretion, with the highest levels in the morning (cf. Berlin, 1986; WHO, 1991). The concentration of mercury in urine varies with changes in diuresis. This may be partly compensated for by relating the mercury to the concentration of creatinine, or by correction of the mercury content to a defined density (usually 1.018 or 1.024) or osmolality, or by relating the excretion to time. Bacterial growth in the samples may cause reduction of mercury into elemental mercury and losses through evaporation (Magos et al., 1964). Loss of mercury has also been reported from refrigerated and deep-frozen samples (Littlejohn et al., 1976). Addition of e.g. nitric acid (Rathje, 1969), sulfamic acid and Triton X-100 (Skare, 1972; Littlejohn et al., 1976), potassium persulfate

(Trujillo et al., 1974), sulfuric acid and Triton X-100 (Littlejohn et al., 1976), strong alkali (Skare, 1972; Littlejohn et al., 1976) or acetic acid (Lo and Arai, 1989) has been suggested to prevent loss of mercury. However, as judged from our own experience (Schütz, unpublished) and from other studies (Least et al., 1974), the mercury level in urine, stored at room temperature without any preservative added, seems to remain fairly unchanged, at least during five days. This contradictory finding may, to some extent, be explained by the fact that a precipitate, containing a considerable fraction of the mercury, is often formed at storage. Mercury may not be fully recovered if the samples are not homogenized by vigorous shaking before analysis (Skare, 1972; Trujillo et al. 1974).

Quality assurance

Because of the risk of analytical errors, it is crucial that every laboratory runs and *reports* a quality assurance program (cf. Friberg, 1988). This should include both internal and external quality control. The internal quality control procedure should include, in each analytical series, a sufficient number of samples with the same matrix as the study samples, and with a relevant mercury concentration. The results obtained are compared with pre-defined levels of acceptability. The external quality control may be assessed by inter-laboratory exchange of samples with (to the laboratory) unknown concentrations, and by analysis of reference samples with well-defined levels.

There are available a long series of reference materials (Toro et al., 1990).

ANALYTICAL PROCEDURES

Although this survey is focused upon methods for analysis of biological samples, it will also, to some extent, touch upon methods so far only described for analysis of aqueous solutions, but which may be applicable to wet digests of biological samples. On the other hand, it is out of the scope of this survey to cover all of the huge number of methods published during the last decades.

Standard solutions

Water solutions of mercury in the μg/L concentration range have been found to be unstable due to absorption to the storage containers and/or evaporation after spontaneous reduction to Hg(0). Pretreatment of glass vessels with an acid-dichromate solution has been reported to considerably reduce loss of mercury from dilute (0.3 μg/L) standards, probably due to saturation of the available adsorption sites with chromium (Litman et al., 1975). Numerous other container treatment procedures and preserving additives have been suggested to avoid loss of mercury from aqueous samples (see section 'aqueous samples').

Methylmercury in aqueous solution in quartz glass containers decomposes rapidly at intense UV irradiation, which is utilized for analytical purposes (May et al., 1987; Morita et al., 1990). The possibility of decomposition of MeHg by daylight has to be considered,

and the solutions should be checked for the presence of inorganic mercury. However, Lansens et al. (1990) found no difference in the decrease rate for MeHg (10 μg/L) between solutions stored at room temperature in clear glass bottles in daylight or in darkness, respectively. They recommended dilute MeHg solutions to be refrigerated in acid-treated containers. Containers of PTFE were found to be superior to glass containers, and showed no detectable loss of MeHg during storage at room temperature for 6 months. These findings seem contradictory to those of Ahmed and Stoeppler (1986), who reported an almost total loss of MeHg from unpreserved aqueous solutions stored for 26 days in Pyrex glass, as well as in polyethene and PTFE containers. The loss of MeHg from solutions in glass and PTFE containers, preserved with HCl (1 %), HNO_3 (1 %), or NaCl (5 %), was 10-20 % during the period. The MeHg concentrations were, however, considerably lower, 0.02-0.4 μg/L, than in the study of Lansens et al. In conclusion, storage of very dilute (< 1 μg/L) MeHg standards should be avoided, and addition of any preservatives should be carefully considered with regard to the decomposing effect on the analyte (see also Aqueous samples section).

Sample preparation

At storage, all biological fluids turn inhomogeneous. It is thus essential to homogenize the samples before analysis by vigorous shaking or by treatment in an ultrasonic bath.

Frequently, when tissue samples come to analysis, the samples are dehydrated to such an extent that results calculated on a fresh-weight basis are unreliable, and the results have to be expressed on a dry-weight basis. However, the drying of tissue samples may involve problems due to the volatility of mercury compounds. Oven-drying of fresh fish tissue may result in loss of mercury (LaFleur, 1973; Kaiser, 1978), even at a temperature as low as 40 °C (deVargas and Romero, 1989). Severe loss of mercury has been reported for samples of plankton and algae dried at 60 °C (Pillay et al., 1971). Iyengar et al. (1978) observed loss of *in vivo* incorporated inorganic [203]Hg from rat liver after prolonged (3 days) drying at 80 °C. At 105 °C, loss was observed also from other tissues except erythrocytes. Drying of brain tissues in vacuum desiccator up to 7 days was reported to give no loss of mercury (Friedman et al. 1974). Drying of hair samples at 105 °C for 1 h seems to proceed without significant loss (Pineau et al., 1990). Drying of plant samples grown in soil containing [203]Hg showed no loss of mercury after 20 days at 50 °C, while about 20 % was lost after 10 days at 105 °C (Semu et al., 1985).

Freeze drying (lyophilization) has, in many studies, been found to cause insignificant loss of mercury, e.g. from fish tissue (Ramelow and Hornung, 1978; deVargas and Romero, 1989; Horvat and Byrne, 1992), human liver (Goeij et al., 1979), and animal tissues containing radioactive ([203]Hg) inorganic (LaFleur, 1973; Iyengar et al., 1978), and methyl- or phenylmercury (LaFleur, 1973). However, contradicting results have also been reported for some types of samples (Holland, 1962; Pillay et al., 1971; Litman et al., 1975; Horvat and Byrne, 1992).

Total mercury determination

Determination of biological samples for total mercury content usually involves some kind of sample digestion prior to the final mercury determination.

Sample digestion

The literature on sample digestion for total mercury analysis is enormous, due to the numerous combinations of digestion acids, alkalies, oxidizing additives, temperature, digestion time, and digestion vessels.

Dry ashing causes a complete loss (Gorsuch, 1959), unless the ashing/pyrolysis is carried out in a closed system containing an absorption solution, e.g. ignition in a Schniger oxygen flask (Pappas and Rosenberg, 1966; Ruzicka and Lamm, 1969; Ure and Shand, 1974; Helsby, 1976), or in a dynamic system, where mercury is trapped, e.g. in potassium permanganate solution (Schütz, 1969; Watling, 1978), on gold film (Lidums and Ulfvarson, 1968; Anderson et al., 1971; Dumarey et al., 1980), or in a liquid nitrogen trap (Rook et al., 1972; Moody and Paulsen, 1988). Loss of mercury has also been reported at dry ashing with excited oxygen in low-temperature ashing equipment (Gleit and Holland, 1962; Pillay et al., 1971).

Wet digestion includes, in the simplest case, only nitric acid (Rathje, 1969; Coles et al., 1985; Lajunen et al., 1985), but mostly some kind of oxidizing reagent mixture is used. Besides the popular mixtures nitric and perchloric acids (Epps, 1966; Skare, 1972; Kaiser et al., 1978; Einarsson et al., 1984; May and Stoeppler, 1984; Beauchemin et al., 1988b) and sulfuric acid with permanganate (Lindstedt and Skare, 1971; Omang, 1971; Stuart 1978a; Velghe et al., 1978a; Lajunen et al., 1985), a large number of other oxidative mixtures have been used, e.g. sulfuric and nitric acids (Coles et al., 1985), and sulfuric acid with hydrogen peroxide (Friend et al., 1977), with chromic acid (Bouchard, 1978), or with dichromate (Landi et al., 1990), sulfuric-nitric acids with persulfate and permanganate (Mottet and Body, 1974), sulfuric-nitric acids with hydrogen peroxide and permanganate (Anal. Methods Committe, 1977), nitric acid with permanganate (Hawley and Ingle, 1975a; Pineau et al., 1990), nitric-hydrobromic acid (Omang, 1973), sulfuric-nitric-hydrochloric acids (Louie et al., 1985), sulfuric-nitric-perchloric acids (Rains and Menis, 1972), and sulfuric-nitric-perchloric acids with hydrogen peroxide (McMullin et al., 1982). Several authors have added vanadium pentoxide to nitric acid (Korunova and Dedina, 1980; Carrillo et al., 1986) or to sulfuric-nitric acids (Egaas and Julshamn, 1978; Dehairs et al., 1982; Marts and Blaha, 1983) to speed up the oxidation. Also, molybdate with sulfuric-nitric-perchloric acids has been used (Munns and Holland, 1971).

In addition to the numerous acidic-oxidative digestion procedures, also some methods involving alkaline solubilization/digestion have been employed. Gage and Warren (1970) decomposed organomercurials in different biological samples by treatment with cysteine in dilute hydrochloric acid (1 h, 100°C), followed by addition of sodium hydroxide. Magos (1971) split the mercury-carbon bond by adding cadmium and sodium hydroxide. Skare (1972) used 1 M sodium hydroxide for solubilization of fish tissue, which was then ox-

idized with sulfuric acid-permanganate. Chapman and Dale (1982) found that alkaline-per-manganate and sulfuric acid-permanganate mixtures, used in sequence, gave a faster digestion, as compared to sulfuric acid-permanganate alone. Alkaline solubilization, with-out use of oxidizing agents, is utilized for specific determination of inorganic mercury, since it does not decompose covalently bound mercury (see sections Inorganic mercury and Differentiation between inorganic and organic mercury).

The power of oxidizing agents is considerably increased with temperature. Digestion at elevated temperatures constitutes, however, a considerable risk of loss of mercury. Thus, wet digestion in open vessels with sulfuric acid at temperatures as low as 80 °C may cause loss of mercury (Lajunen et al. 1985), while at strong oxidizing conditions, a higher temperature may be tolerated (McMullin et al., 1982; Lajunen et al., 1985). Haas and Krivan (1984) found only minor loss of mercury from sample digests containing nitric-hy-drochloric acids and heated to 180 °C, if an excess of hydrogen peroxide was maintained. Data on the loss of mercury during digestion seem, however, to be highly conflicting. Thus, already at 60 °C, significant loss of mercury was reported at digestion with sulfuric-nitric acids, as well as with permanganate - persulfate (Litman et al., 1975). Also, digestion with sulfuric acid-permanganate (50 °C), and nitric-perchloric acids (75 °C) revealed severe loss of mercury from fish tissue labelled in vivo with [203]Hg (Stuart, 1978a). Litman et al. (1975) suggested that the loss of mercury from sample digests containing nitric acid was due to reduction of mercury into Hg(0) by nitrogen tetroxide formed at digestion. Hoover et al. (1971) demonstrated a severe loss of mercury at digestion, when nitrogen oxides were evolved violently, while no mercury was lost when the digestion reaction was slow.

Loss of mercury during digestion can be reduced by choosing long-necked digestion vessels (Kaiser et al., 1978: nitric-perchloric acids at about 200 °C; Marts and Blaha, 1983: nitric-sulfuric acids with vanadium pentoxide up to 200 °C), or by use of covered vessels (May and Stoeppler, 1984; Louie et al., 1985). Losses can also be minimized/eliminated by use of reflux systems (Gorsuch, 1959; Kim and Silverman, 1965; Analytical Methods Committee, 1965 and 1977; Rains and Menis, 1972; Coles et al., 1985). However, consid-erable loss of mercury has been observed also from reflux systems using oxidizing acid mixtures (Hoover et al., 1971; Dumarey et al., 1987; Landi et al., 1990).

Another way to minimize loss of mercury during digestion is the use of heated closed vessels under pressure. The oxidizing power of the digestion reagents is considerably enhanced at elevated temperatures in pressurized vessels, and the reagent volume can be kept low, thus minimizing the reagent blank. The vessels are mostly made of PTFE (Kotz et al, 1972; van Eenbergen and Bruninx, 1978; Stoeppler and Backhaus, 1978; Korunová and Dédina, 1980; Jackwerth and Gomiscek, 1984; Okamoto and Fuwa, 1984; Welz et al., 1984; Vermeir et al., 1988 and 1989; deVargas and Romero, 1989; Templeton, 1989), but also quartz vessels have been employed (May and Stoeppler, 1984). The solubilization time in PTFE vessels in a micro-wave oven can be short (Templeton, 1989, and Schmit et al., 1991: 0.1-0.5 g sample, 2 minutes; Vermeier et al., 1989: 1 g sample, 20 minutes). However, also in the case of closed PTFE vessels, the possibility of loss of mercury has to be considered (Kaiser et al., 1978). Microwave digestion of too large

samples, using nitric acid with hydrogen peroxide, may involve a risk of such a violent, exothermic reaction that emission from the pressurized vessel may occur (Sah and Miller, 1992). The advantages and problems associated with pressure decomposition procedures in trace element analysis was surveyed by Jackwerth and Gomiscek (1984).

In conclusion, considerable discrepancy afflicts reported data on loss of mercury from samples during preparation and digestion. It is, thus, important to adapt the procedure to real samples and to carefully evaluate recovery, matrix effects, and detection limits for these samples. Mostly, such tests are carried out on spiked samples, but a more distinguished approach is to study the recovery through the different steps of the procedure by use of radioactive mercury isotopes. The most reliable data should be those obtained on samples, in which the mercury has been incorporated *in vivo* (e.g. Clarkson and Greenwood, 1970; LaFleur, 1973; Stein et al., 1974; Iyengar et al., 1978; Stuart, 1978a; Semu et al., 1985).

Atomic absorption spectrometry (AAS)

Mercury determination based on the principles of atomic absorption was introduced already during the 1930s for the control of air mercury levels in work places (Woodson, 1939). Most of the methods are based on the measurement of the absorption of the mercury resonance line at 253.7 nm (from a mercury discharge lamp) by ground-state atoms. A few methods have been published where the main mercury resonance line, 185 nm, is utilized. Although this line is claimed to give at least ten times better sensitivity, it is strongly absorbed by oxygen, and the methods have been of little practical importance.

The detection limit depends basically on the technique for transferring mercury from the sample into ground-state atoms in the gas phase, and on the fraction of the atoms, which can be simultaneously introduced in the light path of the AAS instrument. The conversion into the ground-state atoms is achieved by thermal decomposition or by chemical reduction.

Mercury vaporization by heat

The early AAS methods for biological samples utilized the property of mercury compounds to decompose quantitatively into ground-state atoms on heating. A rather frequent technique involved adsorption of mercury from sample digests on cadmium-impregnated asbestos filters, followed by thermal release (Ballard and Thornton, 1941; Pappas and Rosenberg, 1966; Toribara and Shields, 1968). Lindström (1959) aspirated the sample solution into an air-hydrogen flame, Jacobs et al. (1960 and 1961), and Nielsen Kudsk (1965) used thermal decomposition of extracted mercury dithizonate. Wenninger (1965) pyrolyzed organic material in a nitrogen gas stream, Lidums and Ulfvarson (1968) and Anderson et al. (1971) dry-ashed biological samples in an air stream, followed by amalgamation and thermal release. Hayes et al. (1970) injected and pyrolyzed urine on a heated silver gauze. Dumarey et al. (1980) analyzed i.a. reference samples by pyrolysis

and two-stage amalgamation. The detection limits reported were generally in the range 1-10 μg/kg in biological samples.

A somewhat different approach was presented by Yanagisawa et al. (1983), who pyrolysed solid or water-suspended biological materials (a few milligrams) in an argon stream, and destroyed compounds giving background absorption by passing the pyrolysis products through a column with activated charcoal at 1,200 °C. The detection limit was reported as "sub ng/g".

Brandenberger and Bader (1967) electrolysed urine and amalgamated mercury on copper wire electrode. Mercury was released into a gas cell by applying an electric current through the wire. A detection limit of about 0.1 μg/L was reported.

Mercury vaporization by cold vapour (CV) technique
Chemical reduction of ionic mercury. In 1963 Poluektov and Vitkun reported an increase in the sensitivity by about one order of magnitude, when Sn(II)-containing mercury solutions were analyzed according to Lindström (1959). They associated the increased sensitivity with the reduction and release of Hg(0) from the test solution, which was introduced through a spray chamber. Poluektov et al. (1964) presented a "cold vapour" AAS method with the spray chamber and the flame replaced by an aeration vessel, where Sn(II) was added to a defined volume of sample solution, and the liberated Hg(0) was carried by an air stream to a gas cell. The principle of aeration was, in fact, the same as was utilized by Kimura and Miller (1962) for sample clean up prior to colorimetric determination.

Today, CV-AAS, utilizing the unique property of Hg(0) to easily escape from aqueous solutions into the gas phase through aeration, is by far the most common technique for the determination of mercury in biological samples. The CV liberation of mercury from sample solutions is, however, also used in combination with other current quantification techniques, such as AFS, ICP-OES and ICP-MS.

The reduction of the mercury to Hg(0) is generally achieved by addition of an excess of Sn(II) to the sample solution. *Ionic mercury* is reduced practically instantly by Sn(II) in acid (Hatch and Ott, 1968; Rathje, 1969; Kubasik et al., 1972), as well as in alkaline solutions (Magos and Cernik, 1969; Gage and Warren, 1970; Littlejohn et al., 1976; Greenwood et al., 1977), and no *complete* digestion of the organic sample matrix is needed. In fact, the determination may be performed almost immediately after mixing, e.g. of blood or urine, with the digesting and reducing reagents. However, some authors, e.g. Stuart (1978a) and McMullin et al. (1982), recommend a complete oxidation of the organic matrix for better sensitivity and reproducibility.

Further, *organic mercury* compounds with strong covalent bounds, e.g. MeHg, are not reduced by Sn(II). In order to make these compound accessible to reduction with Sn(II), the mercury-carbon bond has to be broken, either through oxidation (see Sample digestion section), by depletion of mercury from its bindings by addition of an excess of competing ions, e.g. Cd(II) (Magos, 1971; Magos and Clarkson, 1972; Greenwood et al., 1977; Tong et al., 1981) or Cu(II) (Margel and Hirsh, 1984; Munaf et al., 1991), or by use of stronger reducing agents (see below).

When using Sn(II), it should be kept in mind, that Sn(II) adsorbs strongly to glass and plastic surfaces and is difficult to remove (Gage and Warren, 1970; Heiden and Aikens, 1979; Kuldvere, 1982; Wigfield and Perkins, 1982; Louie, 1983). Adsorbed traces of Sn(II) may thus result in a premature release of Hg(0) from sample solutions. Large amounts of nitrogen oxides in nitric acid digests can completely oxidize the Sn(II) added, and have to be destroyed. This is usually accomplished by addition of hydroxylamine hydrochloride, but also urea and sulfamic acid have been used (Adeloju and Mann, 1987).

Another frequently used reducing agent is sodium borohydride, $NaBH_4$ (Toffaletti and Savory, 1975; Kaiser et al., 1978; Sharma and Davis, 1979; Mertens and Althaus, 1983; Margel and Hirsh, 1984; Welz and Schubert-Jacobs, 1988; Ping and Dasgupta, 1989; deVargas and Romero, 1989). As compared to Sn(II), borohydride is a stronger reductant, and less interference has been reported from e.g. iodide and selenium (Kaiser et al., 1978), and iodide, antimony, arsenic, and selenium (Mertens and Althaus, 1983) in aqueous solutions. The difference between Sn(II) and borohydride in reducing power is utilized for differentiation between inorganic and organic mercury (see Differentation between inorganic and organic mercury section).

Reduction with sodium borohydride is sometimes carried out on samples without previous digestion. Margel and Hirsh (1984) reported a detection limit of 1-2 μg/L for blood and urine (1 mL sample). Methyl- and phenylmercury compounds were also completely reduced at pH 9.5 and a temperature above 25°C. Copper sulfate had to be added to urine in order to get a satisfactory recovery of MeHg. However, in case of MeHg, Toffaletti and Savory (1975) reported the formation of an unidentified mercury compound, which was aerated from the sample, but was not detected by AAS unless the vapour was heated to about 700 °C.

Divalent germanium has been used as a reducing agent for the determination of mercury in soils and sediments (Zelyukova et al., 1987). In alkaline solution, also organic mercury compounds were reduced. The detection limit can be estimated at about 5 μg/kg.

Hydrazine borane was proposed by Antonovich et al. (1991) as a reducing agent of intermediate strength, as compared to Sn(II) and borotetrahydride, with the possibility to differentiate between phenylmercury and alkylmercury.

Hydrogen peroxide has also been used for liberation of inorganic mercury in alkaline tissue preparations (Konoshi and Takahashi, 1983), but the rection was slow, about 30 min per sample.

A survey of the possibilities of differentiation between inorganic and organic mercury by choice of reagents is to be found in the section "Specification of mercury".

Liberation of elemental mercury from sample solution. The partition coefficient for Hg(0) between the gas phase and aqueous solutions of hydrochloric, nitric, or sulfuric acid at room temperature has been estimated at 0.4-0.7 for acid concentrations up to about 1 M (Koirtyohann and Khalil, 1976; Tong, 1978; Goulden and Anthony, 1980; Lau et al., 1984). In the case of hydrochloric and nitric acids, Tong (1978) experienced a decrease in the partition coefficient with increasing concentrations. Goulden and Anthony (1980) found a

somewhat higher coefficient for alkaline solutions than for acidic ones, and a 7-fold increase when the temperature of the solution was raised from 25 to 90 °C. Carillo et al. (1986) reported a two-fold increase in peak height at raising the temperature from 20 to 75 °C.

The transfer of Hg(0) from the sample solution into the light path is generally accomplished by aeration with a carrier gas, usually air or nitrogen. To avoid excessive foaming during aeration of uncompletely digested sample solutions, antifoaming agents are sometimes used (e.g. tri-n-butylphosphate; Vermeir et al., 1988). Mostly, the carrier gas is discarded when leaving the gas cell, and the mercury is recorded as a peak signal. However, in some methods, the carrier gas is re-cycled through the reaction vessel and the gas cell in a closed circuit, with the detector signal gradually achieving a steady state (Hatch and Ott, 1968; Munns and Holland, 1971; Lajunen et al., 1985; Landi et al., 1990). This approach is somewhat more time-consuming, but is less sensitive to variations in the aeration gas flow or the Hg(0) release rate. The technique is clearly advantageous when the signal reading has to be taken "by hand".

Also, some "static" systems have been applied, where the gas phase, equilibrated with the sample solution in the reaction vessel by stirring, is displaced by water into a gas cell (Clinton, 1974; Chapman and Dale, 1978; Hon et al., 1983). An even simpler arrangement utilizes a disposable plastic syringe (Stainton, 1971) or a glass syringe (Chou and Naleway, 1984) as the reaction vessel, with subsequent injection of the gas phase into a gas cell (Koirtyohann and Khalil, 1976; Gardner and Dal Pont, 1979). The gas cell may also be directly connected to, or integrated with, the reaction vessel, so that Hg(0) is evolved directly into the cell (Tong and Leow, 1980; Bourcier and Sharma, 1981; Lau et al., 1984). The static systems can be made very sensitive, since the gas volume is not diluted with a carrier gas flow.

When using recirculating or static systems, where the time for the UV irradiation of Hg(0) is prolonged, the possible formation of less volatile reaction products, which can deposit in the measuring system (Shimomura, 1989), has to be considered. Intense UV radiation of air is known to produce ozone, which has been shown to react with mercury within minutes (P'yankov, 1949). The mercury levels were, however, high: several milligrams per cubic meter. A considerably enhanced dissolution of mercury in water has been reported already at ambient air Hg(0) levels and slightly elevated ozone concentration (0.2 ppm; Iverfeldt and Lindqvist, 1986).

As for all AAS methods, the sensitivity and the detection limit for CV-AAS depends highly on the fraction of the analyte, which can be brought from the sample into the light path of the instrument *at the same time.*

Factors affecting the detection limit of the CV-AAS technique are:

(1) Sample size.
(2) Aeration gas flow rate and stability.
(3) Shape of the gas cell (optimation of length, volume and gas flow).
(4) Instrumental noise level.
(5) Construction of the reaction vessel.
(6) Temperature of the sample solution.

(7) Sample matrix (viscosity, surface tension, interfering species).

(8) Adsorption of Hg(0) by the construction material of the CV-generating equipment.

(9) Contamination of reagents and the laboratory environment.

The difference in detection limits between optimized and non-optimized equipment might well be one order of magnitude, or more. Thus, by optimizing the above-mentioned factors, a detection limit as low as 1 ng/L (1 mL sample) was reported for aqueous samples (Hawley and Ingle, 1975a). The same detection limit was reported for an automated system used for analysis of natural water samples heated at 90 °C. These methods might well be applied on biological sample digests, but the practical detection limit will probably be impaired, due to contamination from e.g. the digestion reagents. A statistical approach to the optimization was presented by Zacharias and Stratis (1991).

When constructing CV equipment, the choice of materials is of great importance, since Hg(0) may be adsorbed by several of the materials normally used for the conduction of gases (Stuart, 1978b). Daniels and Wigfield (1991) investigated fourteen types of tubing materials, and found that only nickel, Pyrex®, quartz and PTFE did not adsorb Hg(0) significantly, while, polyethylene (low density), PVC, Tygon®, Nalgene®, latex, stainless steel, and a silicon elastomer adsorbed from 20 % up to 100 %.

Improved sensitivity and detection limits were reported by Tanabe et al. (1980) by use of the 185 nm resonance line and an argon-purged monochromator and reaction vessel. The applicability of the equipment for routine analysis is probably problematic.

Interferences in CV-AAS. The quantitation of mercury by CV-AAS may be interferred with by 1) the composition of the sample solution, which affects the release rate of Hg(0), by 2) gaseous compounds, which absorb at 254 nm, and by 3) light-scattering particles in the gas cell.

Differences in the composition of sample solutions, caused e.g. by unspecified, incompletely digested compounds from the sample matrix, may affect the physical properties of the solution (surface tension, viscosity), and, thus, the release rate of Hg(0) and the signal peak height (Munns and Holland, 1971). In such cases, provided that the aeration gas flow is stable enough, the peak area will probably be more reproducible (Wigfield et al., 1981; Wigfield and Eatock, 1987). Equilibration of Hg(0) between the gas and liquid phases through stirring prior to aeration has also been found to give better reproducibility, and, in addition, higher sensitivity (Littlejohn et al., 1976; Ngim et al., 1988). The use of antifoaming agents has been shown to decrease the mercury absorbance peak (Stuart, 1979); this effect should, however, be reproducible.

Numerous observations of chemical interferences on the release of Hg(0) from sample solutions have been reported. Thus, low recovery at reduction with acid Sn(II) has been associated with Au, Pd, Pt, thiosulphate, bromide, and iodide (Lindstedt, 1970), Au, Pd, Pt, Se, and iodide (Ure and Shand, 1974), Au, Pt, and Ag (Koirtyohann and Khalil, 1976), Se and iodide (Kaiser et al., 1978), iodide (Omang, 1973), Se and Te (Suddendorf, 1981), and Sb, Se, bromide and iodide (Lau et al., 1984). Interference at reduction with borohydride has been reported from e.g. Au, Bi, Cu, Pt, Pd, Rh, and Ru (Rooney, 1976), and Cu, Pb, Ni, and Al (Carrillo et al., 1986). Also, reduction with alkaline Sn(II) in solutions

containing Au, Pd, Se, iodide, sulfide, and cystein (Munaf et al., 1989) has been associated with low recovery. There are, however, considerable discrepances in the reported effects of different species, especially of bromide, iodide, and Se. Thus, Lindstedt (1970) found no effect of hexavalent Se (Sn(II), acidic). A quite opposite effect of bromide (Omang, 1973; Lugowska and Rubel, 1982) and iodide (Lugowska and Rubel, 1982), has been reported. Interference from iodide may be diminished, e.g. at reduction with Sn(II) in alkaline medium, as suggested by Lindstedt and Skare (1971). Pratt (1987) found interference from Se(IV), but not from Se(VI) at acidic reduction with Sn(II). Reduction with borohydride seems to give less elemental interferences than does reduction with Sn(II) (Kaiser et al., 1978; Mertens and Althaus, 1983; Welz and Schubert-Jacobs, 1988). A possible explanation of the interference caused by Cu, Au, and perhaps also by some other metals, might be co-reduction and amalgamation (Toffaletti and Savory, 1975; Rooney, 1976).

Yamamoto et al. (1980) reported incomplete recovery of both inorganic and total mercury when the Magos' method (see: 'Differentiation between inorganic and organic mercury') was applied on blood, liver, and spleen of mice, which had been simultaneously injected with equal molar doses of mercuric chloride and sodium selenite. The recovery increased to the expected level when the alkalinized samples were heated to 40 °C for at least 30 min.

The possible interference from Se was checked (Schütz, unpublished) by analyzing blood, plasma, and urine samples spiked with 500 μg Se(IV) per Litre, which is higher than the average levels in most countries (WHO, 1987). No decrease in the recovery was observed for blood and plasma analyzed according to Einarsson et al. (1984), involving digestion with nitric and perchloric acids and reduction with Sn(II), and for urine analyzed according to Lindstedt (1979), involving digestion with sulphuric acid and potassium permanganate. Mercury was also completely recovered from blood-cell samples when added in the form of very finely divided, solid mercury selenide, corresponding to 40 μg Hg/L.

Obviously, the interfering power of the different elements may vary considerably, due to the sample preparation procedure and other species present in the sample. Though the normal levels of the interfering substances are, in most biological samples, probably below the levels associated with serious interferences (Suddendorf et al., 1981; Omang, 1982), the reaction kinetics and recovery of mercury at CV generation has to be checked for each kind of sample analyzed.

Depending on the type of sample and sample preparation procedure, the cold vapour phase may contain extraneous substances, which absorb at the mercury resonance wavelenght. Such substances may be e.g. nitrogen oxides (Adrian, 1971; Hoover et al., 1971; Korunová and Dédina, 1980), acetone (Manning, 1970b), acetone, methylisobuthyl ketone, and hydrochloric acid (Windham, 1972), or aromatic hydrocarbons (Lindstedt, 1970), and chlorine (Bothner and Robertson, 1975). Also, water vapour has been suspected to give rise to background absorption. However, water vapour itself seems to have no significant absorption at 253.7 nm, but spray mist from the reaction vessel, and, probably, fogging of the cell windows, may create a background-absorption effect

(Hoover et al., 1971; Rooney, 1976; Stuart, 1978b). This interference usually disappeared when a desiccant, e.g. ascarite (Omang, 1971), magnesium perchlorate (Hatch and Ott, 1968; Hawley and Ingle, 1975a; Coles et al., 1985), solid sodium hydroxide (Baltisberger et al., 1979), concentrated sulphuric acid (Kothandaraman and Dallmeyer, 1976), molecular sieve 0.4 nm (Margel and Hirsh, 1984), a cold trap (Magos and Clarkson, 1972; Daniels and Wigfield, 1989), or a preheating tube (Christmann and Ingle, 1976; Oda and Ingle, 1981) was inserted between the reaction vessel and the gas cell, or when windowless or heated absorption cells were used (Rains and Menis, 1972; Gilbert and Hume, 1973; Velghe et al., 1977), or the carrier gas was passed *over* the sample solution in the reaction vessel (Pineau et al., 1990). Desiccants for drying should be used with care, as they may cause contamination (Baltisberger, 1974) or decreasing peak height (Christmann and Ingle, 1976; Stuart, 1978b; Gardner, 1980), most probably due to adsorption of mercury and peak broadening.

Enrichment by amalgamation. The detection limit of the CV-AAS technique may be further improved by pre-concentration of the liberated mercury through amalgamation, followed by thermal release and AAS. Mostly, a gold filter is used for the amalgamation (Lidums and Ulfvarson, 1968; Stoeppler, 1983; May et al., 1987; Welz and Schubert-Jacobs, 1988), but also gold-coated quartz-wool (Kaiser et al., 1978), gold-coated Celite[®] (Konishi and Takahashi, 1983; Suetomi et al., 1991), gold-coated sand (Vermeir et al., 1988), and gold-coated glass beads (Baeyens and Leermakers, 1989) are used. Other amalgamating materials used are Au/Pt alloys (Welz et al., 1984), Ag (Watling, 1975; Schroeder, 1982), and, for air sampling, Pd and Cu (Henriques and Isberg, 1975). However, a marked "ageing" effect and increasing breakthrough of Hg has been reported for Ag (Mercer, 1979) and Cu (Henriques and Isberg, 1975). Lee et al. (1989) utilized gold-coated porous graphite in a graphite furnace for amalgamation, and reported a detection limit of about 0.25 μg/kg or 0.1 ng/L (50 mL sample) for solutions. Even two-stage amalgamation has been utilized (Dumarey et al., 1987; Vermeir et al., 1988). Mostly, the detection limits for these techniques seem to fall within 10-100 ng/L sample solution, but values as low as 0.1 ng/L (May et al., 1987), and an absolute mass below 5 pg (Stoeppler, 1983) have been reported for aqeous samples.

Henriques and Isberg (1975) studied the amalgamation of mercury compounds in air with different metals and found that Hg(0) was amalgamated to about 100 % by Au, Pt, Ag, Pd, and Cu. Methyl- and dimethylmercury were also amalgamated to about 100 % with Au, considerably less with the other metals.

It is quite obvious, from our experience, that mercury is effectively amalgamated with Au at temperatures up to 100 °C. According to Welz and Schubert-Jacobs (1988), mercury may be amalgamated with Au even at temperatures up to 250 °C. In fact, the efficiency of the filter may be increased at an elevated temperature, due to a higher diffusion rate of mercury atoms towards the amalgamating surfaces.

The release of Hg(0) from Au has been reported to be dependent on the nature of the carrier gas. Thus, Baeyens and Leermakers (1989) found that argon or nitrogen removed Hg(0) quantitatively from a gold-coated glass-bead filter at about 250 °C, whereas for pure

oxygen, a temperature of more than 300 °C was needed. These data are in some contrast to the regeneration temperature of 150 °C for the Au film of the piezoelectric sensor for Hg(0) in air described by Scheide and Taylor (1974).

By use of an amalgamation step, a decrease in the sample matrix effect upon the instrument calibration (e.g. due to varying release rates for mercury and unspecific UV absorption) can be expected. Thus, calibration may be carried out by use of standards without the sample matrix present, provided that the overall release of mercury is as complete from the sample as from the standard. Further, a high calibration precision by use of known amounts of mercury vapour was reported by Dumarey et al. (1985) and Friese et al. (1990). The latter group reported results for different biological reference samples in close agreement with certified values.

Damage of the amalgamation filter, possibly by other metal hydrides or elemental chlorine, formed at reduction with tetraborohydride, has been observed (Welz et al.,1984; Mertens and Althaus, 1983). This interference was, however, eliminated by allowing the filter to cool to a lower temperature (100 °C, or less) before amalgamation (Welz and Schubert-Jacobs, 1988). For the same purpose, Mertens and Althaus (1983) recommended washing of the reaction gases through a bubbler with Sn(II) in diluted sulphuric acid. Also, inadequate analytical precision at acidic reduction with Sn(II) has been supposed to depend on deterioration of the Au surface by acid fumes and liquid aerosols (Dumarey et al., 1987). The precision was significantly improved by insertion of a bubbler containing KOH with Sn(II), (see also section 4.3.10.).

Flame atomization (F-AAS)

The detection limit for mercury by F-AAS is quite unfavourable, as compared to many other heavy metals. Instrument manuals indicate, at best, about 300 μg/L. Flame AAS suffers from several disadvantages, e.g. inefficient sample uptake through the spray chamber, and background effects from the sample matrix and the flame. Also, interference from Co, which has a resonance line at 253.65 nm, may occur. Consequently, when applying this technique to biological samples, some kind of enrichment has to be made.

Berman (1967) complexed mercury with ammonium pyrrolidine dithiocarbamate (APDC) in urine, blood, and other tissue samples treated with trichloroacetic acid. She extracted the complex into methylisobutyl ketone (MIBK), and reported a detection limit of about 10 μg/L for blood and urine. Mercury, complexed with APDC, can be quantitively extracted into MIBK from hydrochloric acid (at least up to 6 M) and nitric acid (up to 1.5 M) solutions (Brooks et al., 1989).

The detection limit by F-AAS for volatile metals can be improved by use of a *sampling-boat* attachment. By this technique, sample volumes up to 1 mL can be evaporated and then almost instantaneously introduced into the flame and vaporized. This shall be compared with the sample uptake of 0.05-0.1 mL/s, of which only about 10 % reaches the flame through a conventional spray chamber. Mesman and Smith (1970) extracted APDC-complexed mercury from 50 mL aliquots of urine into 5 mL MIBK, and analyzed 0.2 mL of

this extract. A detection limit of about 10 μg/L urine can be calculated, by assuming the same absolute detection limit, as was reported by Kahn et al. (1968) for water solutions.

Grafite furnace (GF-AAS)

Many trace elements in biological materials are today analyzed by GF-AAS, due to the high sensitivity of this technique. In the case of mercury, however, the extreme volatility implies severe loss during the drying/ashing steps (Lendero and Krivan, 1982), which are needed to minimize false absorbance readings by smoke evolved from the organic sample matrix at atomization. In spite of this, several papers have been published on mercury determination by GF-AAS. The general approach has been to minimize the preatomization losses by addition of different "matrix modifiers", which form thermostable mercury compounds. Also, the access to the more powerful Zeeman background correction (ZBGC) has improved the possibilities for mercury determination by GF-AAS (Koizumi and Yasuda, 1975).

Thermal stabilization of mercury in aqueous solutions during drying/ashing has been reported after addition of different matrix modifiers, e.g, nitric acid with ammonium sulfide (Ediger, 1975: up to 300 °C), hydrochloric acid with hydrogen peroxide (Owens and Gladney, 1976: 140 °C; Alder and Hickman, 1977: 155 °C; Kirkbright et al., 1980: 250 °C), and nitric acid with potassium dichromate or Ni, Cu, or sodium sulfide (Kirkbright et al., 1980: 225 °C). Grobenski et al. (1985) reported poor results for real samples when using matrix modifiers and conventional background correction. However, with ZBGC and a graphite furnace with a L'vov platform, heated with 20 μg Pd at 1,000 °C prior to sample injection, they reported acceptable results for digests of different reference materials (fish, hair, and river sediment) with mercury levels of 1 mg/kg and higher. The drying/ashing temperature was, however, not higher than 140 °C. By use of radiotracer technique, Lendero and Krivan (1982) studied the thermostabilizing effects of mineral acids and hydrogen peroxide. They reported insignificant losses of mercury up to 220-250 °C by use of mixtures of hydrogen peroxide with hydrochloric or nitric acid, and acceptable results for a reference hair sample. Quite recently, Ce(IV) has been reported to stabilize mercury up to 260 °C at analysis of water solutions (Mandjukov and Tsalev, 1990).

Fleckenstein (1985) determined mercury in *solid* biological samples (1 mg) by use of a graphite furnace with a graphite sampling boat and ZBGC. He estimated the detection limit at about 0.1 mg/kg. A similar technique was used by Strübel et al. (1990) for the analysis of urinary calculi.

Filippelli (1987) determined organic and inorganic mercury separately by GF-AAS. He extracted organic mercury from biological samples as chloride complexes, according to Westöö (1966) (see: 'Speciation'). The organic mercury was reextracted into a sodium thiosulfate solution and analyzed by GF-AAS. The inorganic mercury left in the water phase was methylated by use of tetramethyltin and analyzed by the same procedure. The author reported no loss of mercury at ashing temperatures up to 300 °C, and recoveries close to 100 % for organic, as well as inorganic mercury added to different biological matrices, including blood. Analysis of a reference sample gave results for total mercury close to the average of other laboratories. Obviously, sodium thiosulfate, which forms very

stable complexes with organic mercury (Schwarzenbach and Schellenberg, 1965), acts as a thermal stabilizer for mercury. The detection limit can be calculated at ca. 5 μg/L for blood and ca. 10 μg/kg for fish tissue.

Also, extraction with chelating agents has been used in the analysis of mercury by GF-AAS. Thus, Takla and Valijanian (1982) used dithizone in chloroform for the analysis of pharmaceutical products (ashing at 220 °C), and Le Bihan and Cabon (1990) used diethyldithiocarbamate in chloroform for analysis of water samples down to 1 ng/L. Grgic et al. (1989) studied the thermal decomposition of mercury complexed with sulphur-containing chelating agents and attributed the enhanced thermal stability of mercury to the formation of mercury sulfide, which was identified by X-ray diffraction analysis.

The *amounts* of mercury detected by optimized GF-AAS techniques are generally smaller than those detectable by most other techniques. The minimum detectable *concentration* is, however, in clear favour of optimized CV techniques, because of the possibility to use considerably larger samples for a single determination.

Atomic fluorescence spectrometry (AFS)

The sensitivity of AFS is directly proportional to the excitation energy, and can thus be enhanced by increasing this energy. As compared to AAS, simpler and more energy-rich excitation sources can be used, since the line width of the resonance lines is of minor importance. The electronic amplification of the detector signal in AFS is simpler, and produces less signal noise than it does in AAS, where the output signal originates from a small difference between two, comparatively high, signals. Also, by a theoretical approach, it has been claimed, that spectral matrix interferences are considerably less in AFS than in AAS (West, 1974).

Early attempts to analyze mercury by AFS utilized acetylene-oxygen or hydrogen-oxygen flames as the atomization source. Using the 253.7 nm resonance line, detection limits of 0.1-1 mg/L were obtained by dispersive AFS (Winefordner and Staab, 1964; Mansfield et al., 1965). A similar detection limit (1 mg/L) was reported by use of non-dispersive ASF (Vickers and Vaught, 1969). A considerably better detection limit, 2 μg/L, was obtained after extraction of mercury as a dithizone complex from aqueous solutions or urine into an organic solvent (Vickers and Merick, 1968). Excitation at 185 nm and measurement of the stepwise-line fluorescence at 253.7 nm, has been reported to give a detection limit of 0.02 mg/L (Kirkbright et al., 1973).

The great advance for AFS in mercury analysis is associated with the CV atomization technique. The sample preparation procedures are the same as for CV-AAS. Most of the methods described below utilize the CV technique for liberation of mercury from the sample solution. Unless otherwise stated, the aeration gas was argon and the mercury fluorescence was measured directly at the outlet of the gas stream carrying the Hg(0) from the reaction vessel into the atmosphere (a "windowless cell").

Thompson and Reynolds (1971) discovered that aeration with argon gave a much better sensitivity than did aeration with air, which causes quenching of excited mercury atoms by oxygen molecules. A detection limit of about 0.5 μg/L was obtained for acidified,

5 mL urine samples. Thompson and Godden (1975) shielded the carrier gas flow with a laminar argon flow, in order to further minimize quenching by air/oxygen. They reported a detection limit of 0.02 μg/L for 1 mL urine samples and a linear range of 0.02-200 ng.

Hawley and Ingle (1975b) optimized, by size and shape, the reaction vessel and the fluorescence cell and reported a detection limit of 0.005 μg/L for standard solutions (1 mL sample), which was, however, higher than the 0.001 μg/L obtained by CV-AAS utilizing the same reaction vessel and a 600 mm absorption cell (Hawley and Ingle, 1975a).

Ebdon et al. (1981) found a smaller illumination angle (45° instead of the conventional 90°) favourable for the minimization of the background signal. Analysis of a reference sample (orchard leaves; argon-shielded cell) gave results in close agreement with the certified value. They considered 0.2-1,000 ng as the practical working range, which corresponds to a detection limit of 10 ng/g according to the sample preparation procedure used.

Caupeil et al. (1976) used a non-dispersive AFS equipment.and reported a detection limit of 0.05 ng per sample, corresponding to a detection limit of 10 μg/kg in fish tissue. Nakahara et al. (1978) reported a tenfold enhancement of the fluorescence signal by use of a non-dispersive system, as compared to a dispersive one, and a linear response from the detection limit of 0.05 up to 1 μg (20 mL sample). Results for waste-water samples corresponded to those obtained by CV-AAS. Another non-dispersive technique was presented by Hutton and Preston (1980), who obtained, with an unshielded carrier gas flow, a background signal equivalent to 5 ng/L (5 mL sample) and, in practice, a detection limit of 40 ng/L, by careful reduction of the stray light. Analysis of 1 mL samples of acid extracts (prepared according to Hatch and Ott, 1968) of sediments and shrimps gave results in agreement with those obtained by an elaborated dispersive CV-AFS method.

Other techniques than CV have been tried for the atomization of mercury from liquid samples. Thus, Lancione and Drew (1985) used a torch of inductively coupled plasma (ICP), but the detection limit was not better than 30 μg/L. It was improved to 0.2 μg/L when mercury was introduced in the plasma by use of a continuous CV-generating system. However, the best detection limit, 0.04 μg/L, was achieved when the plasma was shut off, and the instrument was run in the CV-AFS mode.

Rigin (1990) extracted mercury and other metals from digested samples as di(trifluoroethyl)dithiocarbamates into carbon tetrachloride, separated them by gas chromatography and determined them by AFS after atomization in an ICP. He reported a detection limit of 0.01 μg/kg and acceptable results for reference samples.

In the 1980s, research has been performed on laser-induced AFS (LI-AFS) after electrothermal atomization of metals. No application of this technique for the determination of mercury seems to have appeared, but for several other metals, detection limits 1-2 orders of magnitude lower than for GF-AAS have been reported (Dougherty et al., 1989; Ometto, 1989, general review). However, the application of this technique on mercury in biological samples may cause problems, due to the volatility of mercury compounds (see: 'Graphite furnace').

Generally, low detection limits seem to be obtainable by CV-AFS, and the linear range exceeds that of AAS by at least one order of magnitude. However, the inherent qualifica-

tion of CV-AFS for better detection limits has hitherto shown no clear advantage over CV-AAS in the methods published. The sensitivity of CV-AAS methods can be considerably increased by prolongation/optimization of the absorption cell (e.g. Hawley and Ingle, 1975a). This possibility is restricted in AFS.

Several commercial instruments based on CV-AFS are available.

Atomic emission spectrometry (AES)

During the 1980s, a rapidly increasing number of methods have been published for mercury determination by AES (often called OES = optical emission spectrometry) after excitation/ionization in a gas plasma, usually argon. The plasma source most frequently used is an ICP, but also other kinds of plasma sources are used, e.g. alternating current plasma (ACP), direct current plasma (DCP), and microwave-induced plasma (MIP). AES has a wide multi-element capability; the linear range extends over 4-6 orders of magnitude.

Mercury may be introduced into the plasma torch by nebulization of the sample, by CV technique, or by GF. By direct pneumatic nebulization of acidified (0.5 % HAc) urine samples into an ICP torch, Lo and Arai (1989) analyzed mercury simultaneously with 10 other metals. The detection limit was 20 μg/L. An improvement in the detection limits for some other metals by a factor of 10-20 by use of an ultrasonic nebulizer has recently been reported (Johnson et al., 1989), and should also be true for mercury.

Analysis of samples with complex matrices may be afflicted by severe spectral interferences, due to atomic or molecular emission lines (Schramel, 1988; Olesik, 1991). In case of mercury, the sensitivity can be considerably increased and the relative impact of the interfering elements minimized by use of CV-generating technique. By nebulization of a sample solution, mixed with a reducing agent into a spray chamber, also a fraction of the sample solution is aspirated simultaneously with the mercury vapour into the plasma, and the multi-element capability is maintained.

Kaiser et al. (1978) analyzed biological samples by MIP-AES and AAS, respectively, after CV generation and amalgamation. They obtained results in good agreement in the range 0.5-340 ng/g. A detection limit of about 0.1 μg/kg dry weight for the AES method can be deduced from the minimum detectable amount reported, 0.05 ng. About the same detection limit was obtained by Natarajan (1988) by a quite similar approach, while Nojiri et al. (1986) reported a detection limit of 0.5 pg, corresponding to 0.01 ng/L in a 50 mL water sample. Mitchell et al. (1986) used electrothermal vaporization from a graphite boat for introduction of solid samples, containing microgram amounts of mercury, into a DCP. Costanzo and Barry (1988) employed ACP-AES as a mercury-specific gas-chromatography detector, with an absolute detection limit of about 0.1 ng.

A different approach to the CV generation was utilized by Tao and Miyazaki (1991), who reduced the sample with borotetrahydride in a continuous flow system, and used porous PTFE tubing as a gas-liquid separator. Disturbing gaseous hydrogen and water vapour, diffusing together with Hg(0) and metal hydrides, were removed in a hollow fiber

hydrogen-separation unit prior to MIP-AES. A detection limit of 0.5 μg/L was reported for mercury in standard solution.

Plasma-AES has been utilized for element-specific detection in the specification of mercury compounds by gas chromatography and liquid chromatography (see: 'organic mercury').

Mass spectrometry (MS)

Since the pioneering work by Gray (1975), mass spectrometry of elements ionized in an inductively coupled gas plasma at atmospheric pressure (ICP-MS) has gained a steadily increasing application for trace-element analysis. The determination of mercury by this technique seems to be quite free from interfering polyatomic mass fragments formed by constituents of the plasma and sample matrices, which may disturb the determination of elements with lower mass units (Houk, 1986; Delves, 1988; Lyon et al., 1988; Templeton et al., 1989; Olesik, 1991). However, especially in the case of mercury, several users of ICP-MS have experienced severe memory effects from samples with high mercury levels.

The ICP-MS technique has a high capability of multi-element analysis (Beauchemin et al., 1988a and b; Ridout et al., 1988; Durrant and Ward, 1989; Schmit et al., 1991). It makes it possible to distinguish between stable isotopes and, thus, facilitates stable isotope tracer studies (Delves, 1988), as well as the use of stable isotopes as internal standards in analytical work (Houk, 1986; Longerich, 1989).

From the viewpoint of analytical sensitivity, the splitting of natural mercury into several isotopes is a drawback. The most abundant isotope, ^{202}Hg (30 % abundance), is normally used for quantification. The stable isotope ^{201}Hg (13 %) (Beauchemin et al., 1988a, b, and c; Moody and Paulsen, 1988), but also ^{199}Hg (17 %) (Haraldsson et al., 1989), has been used for "isotope dilution", to increase the analytical accuracy. This possibility is of special interest for monitoring of the loss of mercury during sample digestion (Beauchemin et al., 1988b), or when the solid content between sample solutions differs, which may affect the ionization and, thus, the sensitivity (Houk, 1986; Olesik, 1991).

The detection limit for ICP-MS is highly dependent upon the integration time. At single element analysis, Gray (1985) reported a detection limit of 0.02 g/L for water solutions containing less than 1 % solid matter. Dilution of serum and urine 10 times, and of solubilized/digested tissue 100-500 times (w/v), implies a detection limit in serum of about 0.2 μg/L and in dry tissue of about 2-10 μg/kg. At multi-element analysis, about 10 times higher detection limit is reported (Gray, 1986; Templeton et al., 1989). The detection limit may be improved by use of CV technique for sample introduction (Haraldsson et al., 1989), and further by use of an amalgamation enrichment step (Völlkopf et al., 1990; 3 ng/L). For the CV generation, sodium borotetrahydride has been preferred to avoid severe instrument contamination by Sn (Haraldsson et al.,1989). Recent information from the instrument producers claim detection limits down to ca 1 ng/L in multi-element mode, by use of flow injection accessories and CV generation.

At analysis, biological samples are usually solubilized and nebulized into the plasma torch as dilute aqueous solutions. Protein solutions (e.g. blood plasma) have been ana-

lyzed undigested, after 10-fold dilution (Lyon et al., 1988), but in most studies, the samples have been digested. In some studies on other metals, a severe loss of sensitivity has been reported, due to uncomplete digestion (Ridout et al., 1988; Friel et al., 1990).

The solid content of the samples should preferably be below 0.2 % to avoid clogging of the plasma torch and the interface between the plasma torch and the MS at prolonged nebulization. A higher solid content can be tolerated by use of the flow injection technique (FIA) (Beauchemin et al., 1988c). The signal was, however, suppressed by a factor of 8 at a sodium concentration of 4.3 %. Wiederin et al. (1991) used FIA and direct sample injection (120 μL/min) into the plasma torch for high salt content solutions. They reported a detection limit of 0.1 μg/L for a 20 μL sample. Plantz et al. (1989) separated mercury from solutions of high salt content (urine and seawater) by complexation and column separation, achieving a detection limit of 40 ng/L (0.5 mL sample; ultrasonic nebulization).

Plasma-MS has been utilized as an element-specific detector for specification of mercury (see: 'Solvent extraction', 'gas chromatography', and 'high performance liquid chromatography').

Another technique used for ionization of elements for MS determination is the spark-source technique (SS-MS), which presupposes solid sample preparations. A recent application of this technique on biological samples was described by Moody and Paulsen (1988). To avoid spectral interferences from organic ions, the samples were burnt in an oxygen stream. Mercury was collected in a liquid nitrogen trap, dissolved and coprecipitated with Ag as sulfide. The detection limit was probably in the range 10-100 μg/kg dry matter.

The advantage of ICP-MS lies in the high capability of multi-element analysis, combined with low detection limits. A drawback has been the high cost of the equipment and a rather complicated handling of the earlier instruments. The last generation of instruments is, however, considerably simpler to use due to an essential development regarding instrument performance and computer software for instrument control and data handling.

Neutron activation analysis (NAA)

At irradiation with thermal neutrons, stable isotopes of mercury and many other elements are converted into radioactive daughter isotopes, that can be identified and quantified by high resolution gamma spectrometry. The irradiation is usually carried out in a nuclear reactor with thermal neutron flux densities of 10^{11}-10^{14} cm^{-2} s^{-1}. NAA is well established as a multi-element technique, and has a reputation of good accuracy. Separation and specification of mercury compounds is, however, not possible, since organic mercury turns into inorganic at irradiation (Rottschafer et al., 1971).

The irradiation time usually varies between a few hours and several days. During the irradiation, the samples, as well as standards and reference samples, are enclosed in sealed quartz ampoules, or, especially at shorter irradiation times and dry samples, polyethylene containers or foils. However, irradiation in heat-sealed polyethylene capsules has been reported to cause considerable loss of mercury, especially from standards (Brune

and Landström, 1966; Pritchard and Saied, 1986; review by Zmijewska, 1977), maybe due to reduction of mercury at radiolysis of water (Heydorn et al., 1975). Loss of mercury has been prevented by cooling of the samples during irradiation (Brune and Landström, 1966; Filby et al., 1970).

The radioactive isotopes utilized for analytical purposes are ^{197}Hg ($T_{1/2} = 64$ h) and ^{203}Hg ($T_{1/2} = 47$ d). The natural abundances of the stable parent isotopes, ^{196}Hg and ^{202}Hg, are 0.15 % and 30 %, respectively, but due to a much higher activation cross section and better counting statistics, the analytical sensitivity is considerably higher by use of ^{197}Hg. The gamma spectrometry of ^{197}Hg is, however, heavily disturbed by radioactive isotopes of several common elements in biological samples, such as bromine, phosphorus, and sodium, but severe interference from the less common element samarium has also been reported (Litman et al., 1975). Counting of irradiated samples after varying decay periods is used to discriminate mercury from radionuclides with diverging half-times. In the case of ^{203}Hg, the main interfering element is ^{75}Se (half-life 120 d).

Due to the presence of interfering elements, the determination of mercury in biological materials at low trace levels ($< 10~\mu$g/kg) is usually carried out by use of a "destructive" or "radiochemical" technique (RNAA), which means chemical separation of mercury from other elements with interfering gamma spectra. This separation is generally performed after irradiation. The risk for contamination of the sample with radioactive mercury isotopes is low, while, at the same time, a known, relatively large amount of inactive mercury can be added as a "carrier", and the recovery after separation assessed by an independent, less sensitive technique (e.g. gravimetric determination of electrolytically deposited mercury). The principles for some procedures for the separation of mercury from interfering elements in different types of samples at multi-element analysis have been outlined by Pietra et al. (1986).

There is a large variety of RNAA methods for mercury in biological samples, of which only a few will be mentioned here. Sjöstrand (1964) separated mercury from interfering elements by distillation and electrolytic deposition on a Au foil. He estimated the detection limit for a 0.5 g sample at 10 μg/kg. Kellershohn et al. (1965) determined the recovery gravimetrically (20 mg carrier) after digestion, reduction, and distillation/condensation and reported a detection limit of 0.1 μg/kg. Bayat et al. (1985), and Drabaek et al. (1986), also separated mercury from the sample matrix by distillation, in the former case without mercury carrier. In both cases, a detection limit of 1-2 μg/kg was obtained for 0.1-0.2 g samples and the results for NIST reference materials were close to the certified values. Kim and Silverman (1965) and Monnier and Loeppe (1967) added a droplet of mercury to the irradiated sample to "dissolve" the radioactive mercury by a rapid, heterogeneous exchange reaction (De Voe et al., 1960). The droplet was then separated from the sample and dissolved in acid for radioactivity measurement. Kosta and Byrne (1969) dry-ashed the irradiated sample in a combustion tube and separated mercury from other volatile elements through selective adsorption on a strip of selenium-impregnated filter paper. Pillay et al. (1971) precipitated mercury as the sulfide, and, after dissolution in *aqua regia*, deposited it electrolytically on a Au foil for radioactivity measurement. Rook et al. (1972) and Muramatsu et al. (1988) burnt the irradiated sample, after addition of carrier, in an

oxygene gas stream. Rook et al. collected the mercury in a condenser cooled with liquid nitrogen, and dissolved it in nitric acid for radiometry, while Muramatsu et al. trapped and measured the mercury on activated charcoal. Cresta et al. (1976) used a similar combustion-distillation step and performed the counting on a sulfide precipitate.

Separation of mercury from interfering radionuclides by anion exchange (Dowex-2) has been utilized by e.g. Jones et al. (1971) and Rottschafer et al. (1971). Tjioe et al. (1977) determined mercury among 13 other elements in digested samples after distillation with hydrobromic acid and anion exchange. The detection limit was estimated at about 0.05 μg/kg dry weight. Lin Xilei et al. (1988) separated 13 trace elements, including mercury, in lyophilized human blood serum after digestion with nitric and perchloric acids (Bethge apparatus, carriers added). The elements were group-separated by selective retention on three linked columns containing different inorganic ion exchangers. The results calculated by the k0-method coincided with those obtained by comparison with irradiated standards. Renterghem et al. (1992) determined mercury and 11 other elements in human blood serum. They separated mercury from interferring elements by retention on an ion exchange column (Dowex 1-X8).

Separation by solvent extraction has been employed by e.g. Henke et al. (1968) and Taskeva et al. (1988), who used dithizone, and Zhuang et al. (1989) and Greenberg (1980 and 1986), who used zinc- and nickel diethyldithiocarbamate (DDC), respectively, as complexing agents for extraction of mercury from irradiated, digested samples. Taskeva et al. reextracted mercury and Cu into a water phase, and separated mercury from Cu by a final extraction into toluene-trioctylamine. Greenberg (1980 and 1986) measured the activity of [197]Hg as well as of [203]Hg, and reported results for different reference materials in good agreement with the certified values. Grimanis and Kanias (1982) added 20 mg of inactive carrier, and extracted mercury as mercury iodide into toluene, with back-extraction into an EDTA-ammonia solution. The recovery was determined by re-irradiation and instrumental NAA (see below) of an aliquot of the final sample solution. Lo et al. (1982) separated mercury by solvent extraction with DDC from wet ashed biological samples, prior to irradiation, and thus avoided interfering radionuclides.

Access to high-resolution germanium-lithium {Ge(Li)} detectors, and later high purity germanium (HPGe) detectors, together with better pulse processors and computer programs, has promoted the use of "non-destructive" or "instrumental" methods (INAA), where the irradiated sample is submitted to gamma spectrometry without any chemical separation. The ratio between different photopeaks of ^{75}Se is used for correction of the [203]Hg photopeak. Filby et al. (1970) determined mercury in different biological materials by INAA after a decay period of 3-6 weeks from irradiation, to avoid interferences from [24]Na, [82]Br, and [32]P. The detection limit for mercury in blood was calculated at 3.5 μg/L. Rossi et al. (1976) determined mercury and Se in foodstuffs, blood, and urine, and also in tissues from mercury-exposed subjects by an INAA method, originally developed for water analysis (Clemente and Mastinu, 1974). The results obtained were within plausible ranges. The detection limit was 1 μg/L for urine and 10 μg/L for serum. Yukawa et al. (1980) determined mercury among other trace elements in human tissues by INNA. The mercury levels obtained were, however, rather high, probably due to loss of mercury from the

calibration solutions during infrared evaporation. Ehmann et al. (1982 and 1986) determined mercury among other elements in human brain samples, and later (Ehmann et al., 1987) also in blood, hair, and nails; they used biological standard reference materials for calibration. Pritchard and Saied (1986) studied different method parameters affecting mercury determination in human beard by INAA. To avoid erroneously high results due to loss of mercury from standards during irradiation, they used Bowens cale as a secondary standard for calibration. No loss of mercury was observed from hair samples, orchard leaves, or cale during irradiation (24 h; 60 °C). They reported a detection limit of 10-20 μg/kg. The interference from Se corresponded to about 30 % of the lowest mercury levels, 0.1 mg/kg, and was neglectable at levels above 2 mg/kg.

The classical calibration of NAA methods involves comparison of sample activities with those of co-irradiated standards of the same element. Especially at multi-element analysis, the need for a large number of standards has limited the sample throughput capacity. To circumvent this, alternative calibration techniques have been elaborated. The single comparator method makes multi-element determinations possible, by use of a single element standard (neutron flux monitor). The mass of the analyte is calculated by use of an experimentally determined element-specific factor (k-value), valid for the analytical equipment in question (Girardi et al., 1965; Linekin, 1973; Simonits et al., 1975). Later, a more generalized standardization method, based on accurately determined constants for the active compound nuclei (k_0-factors), and applicable to various analytical equipments, has been proposed (Moens et al., 1984; De Corte et al., 1987).

Other radiometric methods

A rather specific method, based on *substoichiometric isotope dilution analysis* (SIDA), has been described by Ruzicka and Lamm (1969). Dried biological samples (1-2 g) were burnt in oxygen in a Schöniger flask. After extraction with dithizone, and back-extraction in aqueous phase for cleaning up and concentration, a known amount of [203]Hg and a substoichiometric, known amount of dithizone were added. The mercury dithizonate was extracted into carbon tetrachloride and the activity measured. The amount of mercury in the sample thus becomes inversely related to the activity of the organic phase. Interference from other metals, which form less stable complexes with dithizone than does mercury, is eliminated through the substoichiometric amount of complexing agent. Detection limits of 2 μg/kg dry weight for biological samples and 40 ng/L for water samples were obtained. Analyses of reference material were in good agreement with the expected values.

Among other complexing agents for SIDA-analysis of mercury in environmental or biological samples are thioglycolic-β-aminonaphtalide (thionalide) and N,N-di-n-butyl-N'-benzoylthiourea (DBBT). Kanda and Suzuki (1980) used thionalide in chloroform to determine total mercury, and, after separation by extraction, organomercury, in hair. Schucknecht and König (1988) used DBBT in chloroform for analysis of industrial process water; a detection limit of 50 μg/L was reported.

Another analytical use of radioactive isotopes involves *isotope exchange*, which is based on a stoichiometric exchange between stable and radioactive isotopes. E.g. radioactive mercuric mercury (^{203}Hg) in aqueous solution exchanges with Hg(0) in the gas phase, which is in contact with the solution, while covalently bound mercury is not exchangeable (Clarkson, 1969). Norseth and Clarkson (1970) used the isotope exchange reaction to determine inorganic mercury in different tissue homogenates (0.5 M sodium hydroxide and 0.05 M cystein) from rats administered MeHg. In hydrochloric acid (0.1-3 M), also phenylmercury, but not MeHg, undergoes isotope exchange (Stary and Prásilová, 1976a). The technique was utilized by Drabaek and Carlsen (1984) to differentiate between phenylmercury and MeHg in tissue samples. Analysis of MeHg has been performed after extraction of MeHg chloride from hydrochloric acid solution (ca. 3 M) into benzene or toluene, and shaking with an aqueous ascorbic acid (1 %) solution of radioactive ^{131}I. The iodide is insoluble in the organic phase, but exchanges quantitatively with the MeHg chloride (Stary and Prásilová, 1976b; Drabaek and Carlsen, 1984). Interferences from other elements are limited (Stary and Prásilová, 1976b), and detection limits in the low ppb range seem to be obtainable.

X-ray spectrometry

The most important analytical properties of X-ray fluorescence (XRF) and particle-induced X-ray emission (PIXE) are rapidity, nondestructiveness, and multi-elemental capability. Since the methods are only moderately sensitive, they are most useful if, in addition to mercury, other elements are interesting too. Another important property is the possibility of direct analysis, without any sample preparation.

Mercury was included in a thorough review of XRF techniques in clinical studies (Leyden and Noridy, 1977). For normal XRF analysis of 1 g of dried soft tissue, the lower limit of detection was found to be a few mg/kg. For PIXE, the relative detection limits in biological material are in the order of a few tenths of mg/g. For soft tissue analysis, this is normally orders of magnitude too high, compared with "normal" levels of mercury. Thus, the X-ray techniques must be combined with a preconcentration stage. Preconcentration techniques prior to mercury determination means serious risks of losses due to the volatility. To minimize such losses, lenient concentration procedures, e.g. low temperature ashing (Pallon and Malmqvist, 1981), are required.

Traditionally, XRF and PIXE have been applied to "hard" biological materials, e.g. hair, nails, and teeth, which normally contain considerably higher concentrations of mercury than do soft tissues. The X-ray techniques have, in addition, often been used in order to utilize special properties, such as direct analysis and/or high lateral resolution.

Using very tight geometry and excitation by direct brems-strahlung, XRF analysis can be performed with mm-resolution (Jaklevic et al., 1978; Toribara et al., 1982). This technique has been extensively applied to investigations of single hair strands, with particular emphasis to mercury determination. The tragic MeHg poisoning in Iraq in 1971 has produced an extensive data base for longitudinal studies in human hair, which are still under evaluation (Cox et al., 1989). Also, in a continuing investigation of prenatal expo-

sure to MeHg in the Seychelles, hair samples are collected for high-resolution XRF analysis, to study maternal hair mercury concentration during pregnancy, in order to correlate with observed effects.

Attempts have been made to measure mercury in tissue by *in vivo* XRF (Bloch and Shapiro, 1986; Skerfving et al., 1987), but the technique seems to lack adequate sensitivity.

Also PIXE analysis has been carried out with mm-resolution along single hair strands (Li et al., 1984). The analytical capability is similar to that of XRF, with a detection limit of approximately 5 mg/kg of mercury for mm-scanning of a hair (2 h/100 mm). Using ion beams, particular care has to be taken to avoid losses of mercury by evaporation, due to vacuum, radiation damage, and heating. Therefore, the analysis is preferably performed by extracting the ion beam in helium, allowing convective cooling of the irradiated sample, and reducing losses of compounds with a high vapour pressure.

PIXE, being an ion-beam based trace-element technique, is capable of analyzing at a lateral resolution of micrometres (Malmqvist, 1986). This has been used, for example, in a study of mercury migration in teeth with amalgam fillings (Lindh and Tveit, 1980). In a more recent investigation, trace element profiles in human blood cells were determined by microPIXE (Johansson and Lindh, 1987).

Electrochemical methods

Among the electrochemical methods, anodic stripping voltammetry (ASV) and potentiometric stripping analysis (PSA) have been reported to give low detection limits for mercury.

In the case of ASV, a detection limit just below 1 μg/L has been reported for aqueous solutions (Kiekens et al., 1984; Jaya et al., 1985). An early method for biological samples, utilizing a glassy carbon electrode and differential pulse ASV, was suggested by Franke and Zeeuw (1976), but no results were presented. Leu and Seiler (1985) analyzed digested urine by use of a Au electrode. They reported a detection limit of 0.5 μg/L. Liu et al. (1990) determined mercury in digested urine samples by use of a glassy carbon electrode coated with a film of an ion-exchange resin (Nafion-130[®] , Du Pont), containing a metal-complexing Schiff base, N,N'-bis(3-carboxysalicylidene)trimethylenediamine. A detection limit of about 0.1 μg/L urine can be concluded from the data presented. Mannino et al. (1990) determined mercury in digested fish samples by square-wave voltammetry (SWV), using a glassy carbon electrode and standard addition. They reported a detection limit of about 10 μg/kg fresh weight. The results for different fish species were in good agreement with those obtained by CV-AAS.

A quite different technique for the determination of mercury by ASV was described by Scholz et al. (1987). They liberated mercury from sample solutions by a CV technique, amalgamated it on a gold-plated electrode, and determined the amount of mercury by ASV. A detection limit of 30 ng was obtained.

By use of PSA, which is a more recent development in the field of electrochemical methods (review: Jagner, 1982), a detection limit of 45 ng/L has been reported (Huiliang

et al., 1987a). The low detection limit required, however, a rather long electrolysis time (10 min) prior to stripping, and a sequential analysis of the same sample, spiked with mercury, had to be performed for calibration of the electrode. The same procedure, applied on a reference urine sample (51 μg/L) digested with nitric acid and potassium permanganate, gave results in agreement with the certified value (Huiliang et al., 1987b). The electrolysis time was, in this case, 1 min, and the detection limit was probably 5 μg/L.

Application of an electrochemical detector to the determination of organomercurials after liquid chromatographic separation was described by MacCrehan and Durst (1978) (see High performance liquid chromatography section).

The instrumental equipment for these methods is rather cheap, but the application presupposes a proper knowledge on redox reactions, which, probably, has been an obstacle to a wider use of these techniques. Both techniques are, to some extent, capable of multi-element analysis.

Gold-film detectors

Mercury vapour is efficiently absorbed (amalgamated) on Au surfaces (Anderson et al., 1971). The absorption is quite insensitive to other gaseous compounds present in the air (Scheide and Taylor, 1974; Murphy, 1979).

The slight mass increase caused by the absorption of mercury on a gold-plated piezoelectric crystal can be measured as a change in the resonant frequency of the crystal. A piezoelectric detector for mercury in air was described by Scheide and Taylor (1974). Another detector was constructed by Ho and Guilbault (1981), who used it for determination of mercury in water after CV generation. A detection limit of about 1 ng per sample can be deduced, which might also be applied on biological samples.

Further, amalgamation with mercury changes the electrical resistance of Au films. McNerney et al. (1972) described a portable conductometric gold-film detector with a detection limit of 0.05 ng. Murphy (1979) used a commercial gold-film detector and CV generation for analysis of water solutions and acid digests of biological samples. He estimated the detection limit at the ng/L level for water samples, and reported results in close agreement with those obtained by CV-AAS. Ping and Dasgupta (1989) used a similar detector for analysis of mercury in water and urine, following digestion with Fentons reagent, reduction with borohydride, and CV generation. According to the preparation procedure described for urine samples (200 μL), the detection limit corresponds to ca. 3 μg/L. McNerney (1983) demonstrated coincident calibration curves at calibration of a commercial gold-film detector, designed for liquid samples, with mercury vapour and standard solutions, respectively.

When using borotetrahydride for the reduction of mercury prior to amalgamation, care must be taken to eliminate volatile compounds, which can damage the Au film (Mertens and Althaus, 1983; Welz et al., 1984; see also section 'Enrichment by amalgamation').

Speciation

Elemental mercury

Henderson et al. (1974) demonstrated the presence of a small fraction of mercury in urine, that could be determined by CV-AAS without addition of a reducing agent. They supposed that this fraction represented Hg(0). Stopford et al. (1978) also found traces of Hg(0) in some urine samples from workers exposed to mercury vapour. They found no correlation between these levels and mercury levels in the air (personal monitoring). Yoshida and Yamamura (1982) included an amalgamation step and obtained a detection limit of 0.05 μg/L. The Hg(0) levels in urine tended to increase with increasing exposure, but were always below 0.5% of the total inorganic mercury levels. It can not be excluded, that the findings reported above, at least to some extent, are method artifacts. Wigfield and Perkins (1982) concluded that about 1.2 % of Hg(II) in aqueous solutions was converted to Hg(0) by traces of Sn(II), which were strongly adsorbed to the walls of the reaction vessel. Also, possible interactions between Hg(0) and Hg(II), and disproportiona-tion of Hg(I) have to be considered (Toribara et al., 1970; Baltisberger et al., 1979; Pinstock and Umland, 1985).

Inorganic mercury

Inorganic mercury in biological samples may be determined by *selective reduction* to Hg(0), without affecting alkylmercury compounds present. Clarkson and Greenwood (1970) demonstrated the selectivity of Sn(II) for the reduction of radioactive inorganic mercury in slightly alkaline, undigested biological samples. Selective and fast reduction by Sn(II) is also achieved in strong alkaline (Magos and Cernik, 1969; Gage and Warren, 1970), as well as in strong (ca. 16 M) sulphuric acid (Velghe et al., 1978b) solution. Suetomi et al. (1991) determined inorganic ionic mercury by reduction with Sn(II) in aqueous tissue homogenates containing ca. 1 M sulphuric acid and ca. 2.3 M NaCl. The reaction was, however, slow; the release of Hg(0) required 30 min. Also, inorganic mer-cury, but not organic mercury, may be slowly transformed into Hg(0) by hydrogen peroxide in strong alkaline solution (Konishi and Takahashi, 1983).

Some data indicate that the presence of Se may delay the release of mercury from sample solutions at CV-AAS analysis (see: 'Interferences in CV-AAS'). Yamamoto et al. (1980) observed, at determination of total mercury by CV-AAS according to Magos and Clarkson (1972), a delayed and incomplete release of mercury from blood incubated with inorganic mercury and selenite, as well as from some organ tissues from mice, to which these compounds had been administered simultaneously. The recovery of mercury was normalized when the tissue homogenates were heated (40 °C) with an equal volume of 45 % sodium hydroxide solution. Magos et al. (1984) also found that Se, in different forms of bioavailability, administered to rats, affected the determination of total mercury by the above-mentioned method. They determined a "reduction-resistant" fraction of mercury as

the difference between results obtained by NAA and CV-AAS, and accounted the reduction-resistancy to formation of a mercury-selenium compound.

The method of Velghe et al. (1978b) for selective reduction of inorganic mercury with Sn(II) in fish solubilized in sulphuric acid was applied, with some modifications, on blood samples spiked with a large excess (60 μg/L) of MeHg. The results indicated complete recovery of inorganic mercury and no interference from MeHg (<1 % decomposition; Brunmark et al., 1992). Mercury, added in the form of finely dispersed mercury selenide to blood cells, was not detected by this method, while it was completely recovered after digestion (70 °C) with nitric and perchloric acids (Schütz, unpublished. See also section 'Interferences in CV-AAS').

Differentiation between inorganic and organic mercury

Several techniques have been employed for the differentiation between inorganic and organic mercury compounds, e.g. selective reduction of inorganic mercury, selective extraction, steam distillation, ion exchange, gas chromatography, and liquid chromatography.

Step-wise reduction

Magos (1971) introduced a cadmium reagent to break the carbon-mercury bond. Thus, after selective reduction of inorganic mercury by Sn(II), and aeration from the solution, organic mercury can be determined in a second step after decomposition with alkaline Cd. Another ligand used for breaking the carbon-mercury bond is Cu (Margel and Hirsh, 1984; Munaf et al., 1991). Antonovich et al. (1991) suggested selective reduction of inorganic mercury with Sn(II), reduction of inorganic and phenylmercury with hydrazine borane, and reduction of total mercury with Sn(II) after oxidation with bromide/bromate.

The principle of the Magos' (1971) method has gained a wide application for the differentiation between inorganic and organic mercury in biological samples. The method has been modified for higher sensitivity and to fit a variety of biological materials (e.g. Magos and Clarkson, 1972; Ebbestad et al., 1975; Kacprzak and Chvojka, 1976; Littlejohn et al., 1976; Greenwood et al., 1977; Yamamoto et al., 1980; Farant et al., 1981). Oda and Ingle (1981) and Riisgård and Hansen (1990) used in the second step borotetrahydride for the reduction of organomercury compounds. An automated version of the Magos method has been described by Wigfield et al. (1982).

According to Magos (1971), and Oda and Ingle (1981), at alkaline conditions, phenylmercury behaves like MeHg, while, at strong acidic conditions, phenylmercury is reduced together with inorganic mercury (Campe et al.,1978; Rubel and Lugowska, 1980). According to Miller et al. (1958), phenylmercury resists treatment with hot sodium hydroxide and potassium permanganate solutions. Campe et al. (1982) observed that alkylmercury, but not phenylmercury, decomposed slowly in hot (100 °C) alkaline digests of hair samples. To avoid over-estimation of inorganic mercury, due to decomposition of organic mercury, Matsuo et al. (1989) extracted the organic compounds from the sample prior to the determination of inorganic mercury according to Magos (1971).

Solvent extraction

Capelli et al. (1979) used a solvent-solvent extraction according to Westöö (1968) to separate inorganic and organic mercury in fish homogenates. The organic mercury was back-extracted into an aqueous solution containing cystein, and determined by CV-AAS according to Magos (1971). The detection limit can be estimated at about 10 μg/kg. Filippelli (1987) used a similar separation technique for different biological samples and determined mercury by GF-AAS (see: 'Graphite furnace'). Beauchemin et al. (1988c) employed a separation procedure according to Uthe et al. (1972) and determined organic, as well as inorganic mercury successfully in biological reference materials by flow injection ICP-MS. The detection limit can bee calculated at ca. 50 μg/kg.

Steam distillation

Mitani (1976) separated alkylmercury from inorganic mercury by steam distillation of homogenized tissue samples acidified with hydrochloric acid. The distillate was made alkaline and mercury was determined by CV-AAS after addition of copper sulphate and Sn(II). The inorganic mercury in the distillation residue was determined by CV-AAS after oxidation of the organic matrix. The recovery of MeHg and inorganic mercury added to blood and placenta samples was close to 100 %, and the detection limit was about 2 μg/kg. Nagase et al. (1980) applied this technique on river sediment samples. Horvat et al. (1988) reported better than 95 % recovery of MeHg in distillates from a variety of biological reference sample homogenates added with sulphuric acid and sodium chloride. Alkylmercury in the distillate was decomposed by UV irradiation and determined by CV-AAS.

Ion-exchange chromatography

May et al. (1987) extracted MeHg and inorganic mercury from biological samples with hydrochloric acid (in darkness, to avoid decomposition by UV irradiation) and adsorbed inorganic mercury, as $HgCl_4^{2-}$, on an anion-exchange column (Dowex 1xW8). Methylmercury in the effluent was decomposed by UV irradiation and determined by CV-AAS. Inorganic mercury was eluted with nitric acid and quantified by CV-AAS. A detection limit of 0.1 μg/L for biological fluids and 0.2 μg/kg for tissue samples was reported (270 mm gas cell). Phenylmercury decomposed on the column and was determined together with inorganic mercury.

Gas chromatography

Cappon and Smith (1977, 1981, and 1982) determined inorganic and organic mercury in different types of biological samples by use of a modification of the extraction and gas chromatographic procedure for MeHg elaborated by Westöö (1968) (see: 'Organic mercury'). The inorganic mercury, left in the aqueous phase after extraction of organic mercury, was determined after methylation with tetramethyltin, and repetition of the analytical procedure. The analytical recoveries were routinely determined by liquid scintillation spectrometry on small amounts of organic and inorganic [203]Hg added to each sample.

They reported a detection limit of about 1-10 μg/L (kg), depending on the type of sample. A similar separation was described by Filippelli (1987) (see: GF-AAS).

High performance liquid chromatography
Several methods have been described for the determination of inorganic mercury together with speciation of organic mercurials (see below).

Organic mercury

The predominant methods for the specification of organomercurials involve separation by gas chromatography (GC), or liquid chromatography (LC). Most of the GC methods are based on an initial solvent extraction of organomercury compounds as halogenides from the sample matrix. Such extraction was used already by Gage (1961), who determined mercury titrimetrically after reextraction into an aqueous sulphide solution and oxidation into inorganic mercury. LC separation of mercury compounds is mostly preceded by solvent extraction of organic and inorganic mercury from the interfering matrix by use of complexing agents. Different types of detectors, more or less specific, can be used with the GC and LC equipments.

Most methods for specification of organic mercury focus on the determination of MeHg, which is the compound of main interest in biological samples of natural origin.

Gas chromatography
Gas chromatography has been the most frequently used technique for the determination of organic mercury compounds. In conventional GC, the compounds are chromatographed as halogenides, and determined by use of the highly halogene-sensitive electron capture detector (ECD). Better mercury specificity is obtained with detectors based on the principles of AAS, AFS or OES, but, for positive identification of the compounds, mass selective detectors have to be used.

The earliest GC-ECD methods for the specification of organic mercury compounds in biological samples were presented by Kitamura et al. (1966) and Westöö (1966). The Westöö method involved extraction of MeHg chloride into benzene (according to Gage, 1961), after addition of a large excess of hydrochloric acid. Chloride has since then been the most used ligand for the extraction of organic mercury, but later also bromide in sulfuric acid solution (e.g. Uthe et al., 1972; Longbottom et al., 1973; Goolvard and Smith, 1980; James, 1983; Brooks et al., 1986; Brunmark et al., 1992), and iodide (von Burg et al., 1974) has been used. The partition coefficient for MeHg halogenide between the benzene and water phases is increasing from chloride to iodide (Talmi, 1975). In many later methods, benzene has been exchanged for the less toxic toluene (Uthe et al., 1972; Analytical Methods Committee, 1977; Hight and Corcoran, 1987; Rapsomanikis and Craig, 1991). Also, methylene chloride has been used in the first extraction step (James, 1983).

Westöö (1966, 1967, and 1968) reported detection limits in the range of 1 μg/kg by GC-ECD. Comparison of mercury levels in fish obtained by NAA, GC-ECD, and GC with

selected-ion monitoring (GC-SIM, m/z = ^{202}Hg$^+$) revealed coincidence between the results (Johansson et al., 1970). The detection limits were the same for GC-SIM and GC-ECD.

Before injection into the GC system, a clean-up of the sample extract is necessary, since lipophile compounds, which also are extracted into benzene or toluene, contaminate the system. Westöö (1967 and 1968) used a clean-up procedure consisting of an intermediate extraction of MeHg into an aqueous cysteine solution. The applicability of the Westöö methods on fish samples was carefully tested and confirmed by Kamps and McMahon (1972).

An intermediate extraction with cysteine or thiosulfate (e.g. Uthe et al., 1972; Goolvard and Smith, 1980; Cappon and Smith, 1982) constitutes the clean-up step in most GC-ECD methods published later. The thiosulfate reagent is more stable over time. However, interfering peaks, caused by emulsion formation and poor separation at extraction, and impurities in the reagents, have been reported (Jacobs and Keeney, 1974). In some methods, different halogenides are used in the first and the final extraction steps, respectively, with regard to favourable partition coefficients and optimal GC performance (chloride and bromide: Cappon and Smith, 1977; bromide and iodide: Uthe et al., 1972; Goolvard and Smith, 1980).

A modification of the clean-up procedure of the Westöö method was used by Horvat et al. (1990), who absorbed MeHg from the toluene extract on cysteine-impregnated filter paper. After washing of the filter paper with toluene, MeHg was set free by addition of sulphuric acid containing bromide, followed by extraction into benzene. The modification eliminated emulsion formation in the cleaning-up step, and gave cleaner chromatograms. A detection limit of 0.1 μg/kg was reported.

A diverging approach to the cleaning-up problem was reported by Watts et al. (1976) and Hight and Capar (1983), who washed fish homogenate with acetone and benzene prior to addition of hydrochloric acid and extraction of MeHg into benzene, thus avoiding a subsequent clean-up extraction. They reported a quantitation limit of 100 μg/kg and 50 μg/kg, respectively, after concentration of the benzene extract. Methyl- and ethylmercury, added to fish homogenate immediately before washing, was firmly bound to the sample matrix and recovered at analysis. Hight and Corcoran (1987) used a similar approach, but exchanged benzene with toluene, and optimized the extraction conditions. They reported a quantitation limit of 250 μg/kg, without concentrating the toluene extract. The recovery of MeHg seems to be close to 100 % by these techniques. Brooks et al. (1986) added thiosulfate to diluted rat blood and brain tissue homogenates prior to washing with benzene. Then, copper(II) bromide was added, and MeHg was extracted into benzene, which was analyzed without further clean-up extraction.

The extraction steps are associated with loss of analyte (Westöö, 1968; Kamps and Mahon, 1972). In order to increase the extraction recovery, different compounds have been added to the sample homogenate to deplete mercury from "strong bonds" with sulfur in the proteins, e.g. Hg(II) (Westöö, 1967), molybdic acid (Westöö, 1968), Cu(II) (Uthe et al., 1972; Goolvard and Smith, 1980), urea (von Burg et al., 1974), Cu(II) with urea (Cappon and Smith, 1977), and Cu with urea and sodium dichromate (Cappon and

Smith, 1982). Of these ligands, Cu(II) is the most frequently used. The addition of mercury(II) converts any dimethylmercury present into MeHg (Westöö, 1968). The Analytical Methods Committe (1977) recommended solubilization of fish tissue by heating with a sodium hydroxide-sodium sulphate solution prior to acidification and extraction.

The analytical recovery through the different extraction steps has to be checked by analysis of spiked samples, or by addition of a suitable internal standard. Thus, e.g. von Burg et al. (1974) and Cappon and Smith (1977) added radioactive MeHg (^{203}Hg) to each sample to determine the individual recoveries by gamma-counting. Von Burg et al. extracted MeHg iodide into benzene from blood acidified with oxalic acid, and reported "reliable" estimations of 1 ng, or 0.2 μg/L for a 5 mL sample. Cappon and Smith analyzed aqueous homogenates and alkaline digests of different biological materials, and reported a detection limit about 1 μg/kg. The suitability of ethylmercury as an internal standard was demonstrated by Goolvard and Smith (1980) to give "reasonably" accurate GC-ECD determinations of MeHg in human blood at the μg/kg level. Methylmercury has been used as internal standard in experimental studies on ethylmercury (Brooks et al., 1986). Brunmark et al. (1992) used propylmercury as internal standard for blood analysis. They reported a detection limit of 0.5 μg/kg by GC-SIM.

The laborious clean-up procedure may be by-passed by utilizing the volatility of organomercury compounds. Thus, Zelenko and Kosta (1973) added potassium hexacyanoferrate(II) and sulphuric acid to tissue homogenate in a diffusion cell, and absorbed the volatile MeHg cyanide formed on cysteine-impregnated paper, from which it was set free by hydrochloric acid and extracted into benzene. By a microextraction technique (0.15 mL benzene), a detection limit at the 0.1 μg/kg level may have been obtained. A semiautomated headspace technique for tissue homogenates was elaborated by Decadt et al. (1985), and modified by Lansens and Baeyens (1990). They added iodoacetic acid to tissue homogenates in sulphuric acid solution, separated the highly volatile alkylmercury iodides on a packed PTFE column (10 % Alltech-1000 on Chromosorb WAW), with argon as carrier gas, and detected mercury by MIP-OES. A detection limit of about 15 μg/kg tissue was obtained. They observed no column performance degradation caused by sample matrix and solvent impurities (see below).

The need for sample pretreatment can also be reduced by use of mercury-selective detectors, which are less sensitive to interferences caused by sample matrix and solvent impurities than is the ECD. AAS detectors have been used after thermal decomposition of the organic mercury compounds (Bye and Paus, 1979; Dumarey et al., 1982; Robinson and Wu, 1985; Gui-bin et al., 1989; Fischer et al., 1993). with detection limits in the range 4-300 μg/kg. Talmi (1975) demonstrated the advantage of MIP-AES as a mercury-selective GC-detector by analyzing benzene extracts from biological samples without any clean-up. He reported a detection limit of 1 μg/kg for MeHg in fish, and presented results in good agreement with those obtained by GC-ECD. Panaro et al. (1987) interfaced a simple, home-made GC, with direct current plasma AES to obtain better specificity, but the sensitivity decreased considerably, as compared to conventional GC-ECD. Quimby and Sullivan (1990) developed a MIP-AES especially for GC and reported a detection limit for mercury of 0.1 pg/s. Berman et al. (1989) determined MeHg in reference materials,

after initial extraction into toluene, and quantification by three different techniques: GC-ECD, CV-AAS, and ICP-MS. By ICP-MS, the detection limit was 17 μg/kg, while that obtained by GC-ECD was 4 μg/kg.

For the GC separation of organomercury compounds, packed as well as capillary columns have been used. Westöö (1966; 1967) used packed stainless steel columns with Carbowax 1500 and 20 M on Teflon 6 (35/60 mesh) and Chromosorb W (AW-DMCS). In later papers (Westöö 1968; Albanus et al., 1972), glass columns with phenyl diethanol-amine succinate, as well as Carbowax 20 M, were used. Glass columns were also used by e.g. the Analytical Methods Committe (1977; Carbowax 20 M on Chromosorb G), Cappon and Smith (1977; OV-17 on Chromosorb W-HP) and Horvat et al. (1988; DEGS-PS or Carbowax R 20 M on Supelcoport and PEGS on diatomite). The column used by Cappon and Smith was reported to be stable for at least 5 months.

The identity of MeHg in fish was confirmed by Jensen and Jernelöv (1969) by GC-MS, using a packed column. In a similar chromatographic system, Johansson et al. (1970) reported substitution of the halogenides: after injection of MeHgCl, the formation of MeHgI was confirmed by MS. No chromatographic separation between the MeHgCl and MeHgI peaks was obtained. Also, when MeHgI was injected, the formation of MeHgCl was found. With this exception, no destruction or transformation into other compounds took place.

Capillary columns have been employed by several authors. Thus, support-coated open tubular capillary columns, with a mixture of m-bis(m-phenoxy-phenoxy)benzene and Apiezion L as stationary phases, were used in combination with helium MIP-OES by Wasik and Schwarz (1980). Capillary columns coated with Superox 20 M have been demon-strated to give symmetrical peaks for organomercury compounds at trace levels (Dumarey et al., 1982). However, only dilute standards were investigated. Glass capillary columns with OV-275 as the stationary phase were demonstrated to give good column efficiency, without adsorption or degradation at the pg level (Brooks et al., 1986). Unpolar and medium polar stationary phases, such as OV-1, SE-52, OV-1701, and Carbowax 20 M, were also studied. When determining amounts less than 10 ng, the peak shape deteriorated and column adsorption was seen. Gui-bin et al. (1989) compared different capillary columns and found an OV-17 column (12 m, 0.3 mm) favourable for the separation of alkyl- and phenylmercury compounds added to fish homogenate.

Improved column performance after conditioning by repeated injections of a concentrated solution of Hg(II)Cl has been reported, e.g. by O'Reilly (1982) for diethylene glycol succinate-packed columns, and by Hight and Capar (1983) and Horvat et al. (1988) for glass columns packed with DEGS-PS on Supelcoport. O'Reilly reported an absolute detection limit of 0.2 pg MeHg (GC-ECD).

Decrease in peak heights, due to contamination of the ECD foil, was experienced after injection of a large number of samples (Jacobs and Keeney, 1974). Therefore, standards had to be injected with every few samples. Less contamination of the detector was experienced when using [63]Ni detectors, instead of ^{3}H ones; the higher operating temperature of the [63]Ni detector decreased the contamination.

The chromatographic properties of the polar monoalkymercury halogenides may be improved by derivatization into dialkylmercury, which has only seldom been employed in

GC analysis. However, Kanno et al. (1985) determined MeHg in environmental samples as dithizone complexes by GC-ECD. The samples were treated with an ethanol solution of potassium hydroxide and, after acidification, extracted with benzene-dithizone. The mercury complex was extracted into aqueous ethanol containing sodium sulfide. After acidification, the excess of sulfide was removed by air bubbling, MeHg was again extracted into benzene-dithizone, and analyzed by GC. The detection limit was 0.5 μg/kg.

Bloom (1989) digested fish samples in a potassium hydroxide-methanol solution and converted MeHg and Hg(II) into highly volatile methylethyl- and diethylmercury, respectively, by reaction with sodium tetraethylborate. The mercury derivatives were aerated from the sample solution and trapped on a graphitic carbon column. The compounds were transferred by thermal desorption onto a GC column cooled with liquid nitrogen, chromatographed at 180 °C, and determined by AFS after pyrolysis at 900 °C. The absolute detection limit was about 1 μg MeHg per kilogram. A similar procedure was described by Fischer et al. (1993), who trapped the mercury derivatives directly on a GC column and detected mercury by AAS. They reported a detection limit of 4 μg/kg dried fish and results for marine standard reference materials in good agreement with certified values.

Rapsomanikis and Craig (1991) extracted MeHgCl from fish muscle homogenate into toluene. After back-extraction into a sodium thiosulphate solution, the sample was evaporated to dryness, redissolved in 0.1 M HCl and treated with sodium tetraethylborate (dissolved in ethanol). After GC separation, the dialkylmercury was pyrolysed in a quartz cell and determined by AAS. The detection limit was 120 μg/kg.

Brunmark et al. (1992) determined MeHg in blood by GC-MS after derivatization, using propylmercury bromide as internal standard. The alkylmercury compounds were extracted into toluene, and back-extracted into a 50/50 ethanol/water solution containing thiosulfate. After addition of sodium bromide, they were again extracted into toluene, and methylated by the addition of a diethylether solution of diazomethane. The dialkylmercury bromides showed no adsorptive behaviour on a fused silica capillary column with chemically bonded unpolar stationary phase (DB-5). The m/z = 215 fragment of dimethylmercury bromide and the molecular ion of propylmethylmercury bromide, m/z = 338, were monitored. The detection limit for MeHg in human blood was ca. 0.5 μg/L.

Bulska et al. (1992) extracted methyl- and inorganic mercury, as diethyldithiocarbamate complexes, from blood into toluene. After buthylation by use of Grignard reagent, the mercury species were separated by capillary GC and determined by MIP-OES. The detection limit was reported at 0.4 μg/L.

High performance liquid chromatography

MacCrehan and Durst (1978) developed a method for the determination of methyl-, ethyl-, and phenylmercury, based on reverse phase LC and differential pulse electrochemical detection. Fish samples were analyzed after alkaline hydrolysis, acidification with hydrochloric acid, and extraction into toluene. A detection limit of 2 μg/kg was reported. The result obtained for a reference sample (NIST RM-50 Albacore tuna), 0.93 mg/kg, was in very close agreement to the certified total mercury level. Lajunen et al. (1984) and

Langseth (1986a) extracted diethyldithiocarbamate complexes of organic and inorganic mercury at pH 9.5-9.7 into chloroform, and studied different reverse-phase columns and mobile phases for the separation. Langseth (1986b) extracted inorganic, methyl-, ethyl-, and phenylmercury compounds as dithizone complexes into chloroform, and determined them colorimetrically (475 nm) after evaporation to dryness, dissolution in methanol, and separation by reverse-phase high performance LC (HPLC). She reported a detection limit of 1.5 μg/L in urine. The method was also applied to the determination of organomercury in tomatoes.

Krull et al. (1986) combined HPLC with CV-ICP-AES and reported detection limits for different mercury compounds between 32 and 62 μg/L in the injected sample. Thompson and Houk (1986) combined sample flow injection with ion-pair reversed-phase HPLC and ICP-MS for multi-element analysis and reported a detection limit of 150 μg/L. Bushee (1988) extracted inorganic and alkylmercury compounds from fat-free tissue samples with toluene, and back-extracted the mercurials into an aqueous mobile phase consisting of ammonium acetate (0.06 M), acetonitrile (3 %) and the complexing agent 2-mercaptoethanol (0.005 %). After separation by LC, and post-column CV generation with borotetrahydride, mercury (^{202}Hg) was determined by ICP-MS. The detection limit can be calculated at 25 μg/kg dry matter for MeHg and 50 μg/kg for ethylmercury and inorganic mercury. For a reference sample (NIST RM-50 Albacore tuna), she reported an average of 873 μg/kg, which was within the expected range, 760-890 μg/kg. Shum et al. (1992) separated mercury compounds by microbore LC, and introduced the sample flow (100 μL/min) through a direct injection nebulizer into an ICP-MS. The detection limit was 4 μg/L for inorganic mercury in urine.

Munaf et al. (1990) utilized microcolumn technique for preconcentration and LC separation of mercury compounds in waste water samples, using cystein-acetic acid as the mobile phase. After separation, the mercury compounds were digested "on-line" with peroxodisulphate at room temperature, using Cu(II) as a catalyst. The mercury was then reduced by alkaline Sn(II) and determined by CV-AAS. The detection limit was calculated at 5 ng/L.

AUTOMATION OF ANALYSIS

Various automated/semiautomated techniques for mercury analysis have been introduced during the last decades. In the case of biological samples, the sample preparation and digestion steps are difficult to automate, and require, as a rule, manual handling. The processing of digested samples and the quantification and evaluation steps may, however, be highly automated by microprocessor control, saving manpower and, probably, improving the precision. Several metal-hydride generation systems with automatic samplers are commercially available and are immediately applicable to CV-AAS, CV-AES, CV-AFS, and plasma-MS. Also, commercial automated CV-amalgamation equipments are available. In addition, most GF-AAS, GC, and LC/HPLC instruments are today attainable with automatic samplers. However, there are also many automated equipments, which

have been individually constructed, mostly by use of conventional techniques for sample changing and transfer of sample solutions and reagents.

It is beyond the scope of this review to try to validate the different technical approaches; only a few references will be mentioned:

A semiautomated method for acid-digested urine samples was described by Lindstedt and Skare (1971). The application of the method on other types of biological samples was reported by Skare (1972). CV-AAS methods for digested samples have also been elaborated by Armstrong and Uthe (1971), Koirtyohann and Kalil (1976), Velghe et al. (1978a), Matthes et al. (1978), Peter and Strunc (1984), and Einarsson et al. (1984; extensively evaluted by Vesterberg, 1991). The use of the Magos reagent may reduce the need for sample digestion, and, thus, allow a higher automation (Coyle and Hartley, 1981; Wigfield et al., 1982).

When the sample matrix is more easy to destroy, as in case of natural waters, waste waters and urine, the digestion step can also be automated. Fully automated CV-AAS methods for analysis of natural waters have been described (e.g. El-Awady et al., 1976; Anderson, 1984; Pratt and Elrick, 1987). The methods include oxidation with potassium persulfate at 95-100 °C; in the latter case also potassium dichromate was added. Acidic reduction with Sn(II) was used. Goto et al. (1988) analyzed waste-water samples after catalytic oxidation with potassium persulfate and Cu(II) at room temperature, followed by alkaline reduction with Sn(II). A fully automated analysis of urine, including digestion with Fenton's reagent and borhydride reduction, was reported by Ping and Dasgupta (1990). The detection limit for a 200 μL sample was 2.5 μg/L.

An automated CV-AFS method for the determination of inorganic and organic mercury, respectively, in water samples was described by Morita et al. (1990). After reduction with Sn(II), Hg(0) was separated from the sample solution by diffusion through a porous PTFE tubing. Total mercury was determined after decomposition of organic mercury by UV irradiation. Goulden and Anthony (1980) used different reagent mixtures for chemical specification of inorganic, aryl- and alkylmercury by CV-AAS, with a detection limit of 1 ng/L.

A reversed-phase LC method for the determination of mercury simultaneously with other metals (as dithiocarbamates), utilizing electrochemical and spectrophotometric detection, was applied to industrial effluents by Bond and Wallace (1984). See also Munaf et al. (1990) in the section 'High performance liquid chromatography'.

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Abbreviations

AAS   Atomic absorption spectrometry
ACP   Alternating current plasma
AES   Atomic emission spectrometry
AFS   Atomic fluorescence spectrometry
APDC   Ammonium pyrrolidine dithiocarbamate
CV   Cold vapour
DCP   Direct current plasma
GC   Gas chromatography
GF   Graphite furnace
HPLC   High performance liquid chromatography
ICP   Inductively coupled plasma
INAA   Instrumental neutron activation analysis
LC   Liquid chromatography
MeHg   Methylmercury
MIBK   Methylisobutyl ketone
MIP   Microwave induced plasma
MS   Mass spectrometry
NAA   Neutron activation analysis
OES   Optical emission spectrometry
RNAA   Radiochemical neutron activation analysis
SIDA   Substoichiometric isotope dilution analysis
ZBGC   Zeeman background correction

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Chapter 21

Nickel

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INTRODUCTION

Nickel is a Group VIII transition element of atomic mass 58.62. Metallic Ni is lustrous, hard and ferromagnetic. By far the most common oxidation states are Ni^0 and Ni(II), although Ni(I)-Ni(IV) participate in biological redox reactions (Walsh and Orme-Johnson, 1987) and higher oxidation states occur in simple compounds (e.g. $Ni(IV)S_2$). A number of Ni compounds are in common use that have mixed formal oxidation states, such as Ni_3S_2. Salts of divalent Ni are water soluble, and hexaquo Ni exists in dilute aqueous solution up to about pH 8 without appreciable hydrolysis (Baes Jr. and Mesmer, 1986). Ni has 5 stable isotopes [58 (68%), 60 (26%), 61 (1%), 62 (4%), 64 (1%)] and several radioisotopes; ^{63}Ni $(\beta^-, 92$ y) is the most useful radiotracer. Ni comprises about 0.008% of the earth's crust by weight (Mastromatteo, 1986), making it the 24th most abundant element, above Cu, Pb and Zn. Commercially important ores include Ni/Fe mixed oxides, Ni magnesium silicate, and Ni sulfides.

About 800,000 tonnes of Ni are mined and processed annually, the major producers being Canada and the USSR (Chamberlain, 1988). Stainless steels and steel alloys account for about half the world's Ni consumption, the rest being used for nonferrous and high-temperature alloys, electroplating, and in smaller quantities for ceramics, magnets, batteries, catalysts and salts (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1990).

BIOLOGICAL AND ENVIRONMENTAL SIGNIFICANCE

Nickel occurs as a cofactor in four enzymes known to date (Walsh and Orme-Johnson, 1987). Ureases from plant and animal sources use two Ni atoms as Lewis acids, a role more typical of Zn. In the other three enzymes, all bacterial, Ni is redox active. In methyl coenzyme M reductase of methanogenic bacteria, Ni is found in a tetrapyrrole (factor F_{430}) and cycles between Ni(II) and Ni(I). Many bacteria contain Ni-dependent hydrogenase(s) and methanogenic and acetogenic bacteria have a specific Ni-containing CO

dehydrogenase. These latter enzymes also contain iron-sulfur clusters and display a complex redox activity that may involve all 4 oxidation states Ni(I)-Ni(IV) (Walsh and Orme-Johnson, 1987). No Ni-containing enzymes have been isolated from tissues of higher organisms. The essentiality of Ni for humans remains unproven and somewhat controversial. Thus the importance of Ni in human health is almost exclusively that of a toxic element.

The toxicity of Ni-compounds varies widely. Soluble Ni salts are rapidly excreted by the kidney with few acute effects (Templeton, 1992) and fatal poisoning with Ni salts is extremely rare, whereas volatile Ni carbonyl is lethal to rats at concentrations as low as 0.2 mg/L (LC_{50} for 30 min exposure) (Sunderman, 1989). IARC (1990) has recently concluded that Ni sulfate, and combinations of Ni sulfides and oxides encountered in the refining industry are carcinogenic to humans, and Ni compounds are now considered to be Group 1 carcinogens. There is insufficient evidence to classify metallic Ni as carcinogenic. Ni is an allergen, and eczematous contact dermatitis is the probably the most common and important health problem in Ni-related industries. Occupational exposure is transdermally and by inhalation and ingestion. Significant exposures occur in Ni mining, refining and smelting industries, stainless steel and Ni alloy production, electroplating, welding, and the production of batteries, catalysts and paints.

Significant acute or long-term effects of Ni arise almost exclusively in an occupational setting. With the exception of allergic contact dermatitis, the effects of chronic environmental exposure of the general population are not well understood. The prevalence of Ni sensitivity is about 1% in males, and as high as 15% in females (Menné et al., 1982; Grandjean et al., 1989), making it perhaps the leading cause of contact dermatitis in women. In industrialized countries, the daily intake of Ni is about 100 μg, mostly from food and water (Grandjean et al., 1989). Various unpolluted water supplies generally have Ni concentrations in the range of 1 to 50 μg/L (Stoeppler, 1980), but this can increase to the mg/L level in industrially polluted waters. The Ni content of the diet is quite variable. While meats have Ni concentrations in the range 0.06 to 0.42 mg/kg, the corresponding level in fruits and vegetables is 0.05 to 9.0 mg/kg. Nuts, spinach, tea leaves, cocoa, soy products and oatmeal are especially rich in Ni, and diets high in these foods can produce significant elevations in urinary Ni excretion (Nielsen et al., 1990). Ni ingested with water by fasting volunteers was about 27% absorbed in the gut, although less than 1% of the Ni in food is generally absorbed (Sunderman Jr. et al., 1989). Inhalation of Ni depends on geographic location. In the U.S., rural air typically contains 6 ng Ni/m^3, while in urban air the concentration is about 25 ng/m^3, and up to 170 ng/m^3 in some industrial centers (Tsalev and Zaprianov, 1984; Sunderman Jr., 1988).

Biological monitoring is mainly carried out for acute exposures in the workplace, and most typically involves urine and serum collection. Although the Ni content of these fluids does not in general indicate specific health risks, biological tolerance values of 30 μg Ni/L in urine and 8 μg Ni/L in serum have been recommended (Sunderman Jr., 1988). These values are at least and order of magnitude higher than those found in individuals without specific sources of excessive exposure.

ANALYTICAL CONSIDERATIONS

The natural level of Ni in many biological samples dictates extreme caution to avoid sample contamination during all phases of sample collection, storage and processing for analysis. For example, Ni in human serum is probably in the range of 0.1 μg/L (Sunderman Jr. et al., 1984; Christensen and Pedersen, 1986; Nixon et al., 1989), and accurate measurement at this level requires thorough implementation of the procedures described in earlier chapters on sampling and sample treatment. Here we reiterate certain features of these procedures as they apply specifically to Ni. Considerations pertinent to Ni when choosing human subjects from a reference population have been described elsewhere (Templeton, 1993).

As noted above, the atmospheric content of Ni is geographically variable, and the nature of Ni particulates will depend in part on local industrial activity. Some samples (e.g. leaves) will be exposed to ambient air, and their Ni content will appropriately reflect this exposure up to the time of processing. The majority of samples, however, are collected invasively by biopsy or venipuncture, and must subsequently be protected from atmospheric contamination. For determination in the sub-μg/L level, as in serum for example, this is generally thought to necessitate handling in clean-room facilities (Sunderman Jr. et al., 1988; Nixon et al., 1989).

Stainless steel collection devices, including needles for blood collection, biopsy needles and scalpel blades, pose a serious risk of Ni contamination, due to the Ni content of various steels. Authors frequently mention the use of silanized needles, implying that

TABLE 1

LEACHING OF Ni FROM STAINLESS STEEL COLLECTION DEVICES DURING SUCCESSIVE SAMPLING. From Versieck et al. (1982).

Device	Sample	Sample number	[Ni]	Units
Stainless steel needle	20 mL Blood	1	75	μg/L
		2	10	
		3	8.5	
		4	13	
Menghini biopsy needle	Liver biopsy[*]	1	6.2, 12.0	mg/kg
		2	5.1, 1.2	
Surgical blade	Liver wedge[*]	1	0.015, 0.064	mg/kg
		2	0.0023, 0.0049	

[*] Duplicate specimens

this should render the surface of the steel sufficiently hydrophobic to prevent leaching. It must be noted, however, that many commercially available silanized needles (e.g. Becton-Dickinson No. 5175 hypodermic needles) are spray-coated on the *outside* of the barrel only, for purposes of lubrication and patient comfort. An indication of the potential leaching of Ni from steels by blood and tissues is given in Table 1. These data were obtained by neutron activation analysis (NAA), after irradiation of the devices (Versieck et al., 1982), and so may ovestimate the problem as a result of damage to the steel surface. However, they suggest extreme caution in interpreting results obtained after collection with such

TABLE 2

LEACHING OF Ni FROM PLASTICS BY 7 DAYS OF EXPOSURE TO 1:1 ACID:WATER AT ROOM TEMPERATURE (80°C FOR TEFLON)

Values of Ni leached are in ng/cm^2. From Moody and Lindstrom (1977).

Acid	Teflon FEP	Polyethene (linear)	Polyethene	Polycarbonate
HCl	0.8	0.8	0.3	0.3
HNO_3	2	1.6	0.5	0.7

TABLE 3

AVERAGE CONCENTRATION OF Ni IN DOUBLY DISTILLED ACIDS AND SOME COMMERCIAL NITRIC ACIDS

Non-commercial acids were purified by sub-boiling distillation in a quartz still and collected in Teflon bottles. Both quartz and Teflon had experienced several years of continuous leaching through use. Taken from Moody and Beary (1982). For the commercial nitric acids are shown the maximum Ni concentration as specified by the manufacturer for the currently available product.

Acid (2 x distilled)	[Ni] (μg/L)
HCl	0.12
$HClO_4$	0.37
HF	0.45
H_2SO_4	0.12
HNO_3	0.08
HNO_3 (Merck; "Supra-Pur")	2 max
HNO_3 (Baker; "Instra-analyzed")	3 max
HNO_3 (BDH; "Aristar")	5 max

materials. Teflon and plastics represent alternatives, but the need for careful acid washing of all material coming into contact with the sample must be stressed. For example, Nixon et al. (1989) have demonstrated serious contamination of serum samples from the use of unwashed plastic pipette tips. Reagents are of course another source of contamination, and in general the fewer additions that must be made during sample preparation the better. It has been helpful in the measurement of Ni in body fluids that atomic absorption spectrometry with graphite furnace (GF-AAS) can be carried out after minimal processing (see below). On the other hand, it is fortunate that tissue samples requiring extensive digestion procedures generally have higher Ni contents and are less prone to serious contamination errors from ultra-pure reagents. The Ni contents of some doubly distilled acids are shown in Table 3, as well as the Ni content, specified by the manufacturer, of several commercial ultra-pure acids. In particular, the benefits of a nitric acid matrix for analysis of Ni by GF-AAS warrant attention to the Ni content of commercial sources.

Any serious effort to determine Ni in biological materials demands an ongoing assessment of the accuracy of the method by using carefully matrix-matched, certified reference materials. A range of such materials is available with certified or suggested values for Ni (see Chapter 11 on "Reference materials for trace element analysis", by R. Parr, in this book), but a serious problem remains. In most cases, the certified values are much higher than the Ni concentrations that the analyst is required to measure, and so the reference material is inadequate for method evaluation. This situation is beginning to be rectified by the important work of Versieck and colleagues (Versieck et al., 1988) with the introduction of a second generation reference material for human serum that has values of many elements closer to the expected level. The best estimate of the Ni content of this material, based on GF-AAS and voltammetry carried out in four independent laboratories, is 0.23 μg/L.

ANALYTICAL METHODS

For the practical analysis of Ni in biological materials, GF-AAS techniques are by far the most important, and for biological fluids demand only very simple steps for sample preparation. Voltammetric methods can provide lower detection limits in specially prepared samples, while less sensitive methods such as inductively coupled plasma-atomic emission spectrometry (ICP-AES) may be useful in multielement protocols with tissues and other solid samples.

Atomic absorption spectrometry (AAS)

The last two decades have seen improvements of about four orders of magnitude in the sensitivity of atomic absorption techniques, due first to substantial improvements in graphite furnace atomization techniques and then to the introduction of Zeeman background correction. Flame AAS (F-AAS) methods are mainly of historical interest for all but the highest concentrations of Ni in biological solids. Furthermore, the pre-concentration and extraction procedures formerly required for determination of Ni in fluids can now

TABLE 4

SELECTED PROCEDURES FOR Ni DETERMINATION IN BIOLOGICAL SAMPLES BY FLAME AAS

Tissues and faeces (Horak and Sunderman Jr., 1973; Nomoto and Sunderman Jr., 1970) - 10 mL of a homogenate (20 g/100 mL) of lung, liver or muscle is placed in a 125 mL Erlenmeyer flask. Add 10 mL H_2SO_4:HNO_3 (1:5 v/v, heat gently with swirling, cool, add 2.0 mL H_2SO_4:HNO_3 mixture with 0.5 mL $HClO_4$, and heat until white fumes of SO_3 appear. This is repeated until the samples are clear and nearly colourless in a volume of < 2 mL. The solution is acidified with 5 mL dilute HCl, interfering Fe is extracted with methylisobutyl ketone (MIBK), and then 3 mL of a Ni-ammonium pyrrolidinedithiocarbamate complex is extracted into 3 mL MIBK for analysis. Faeces from a pooled 3-day collection is placed in a 2 L plastic jar containing 500 mL water, weighed, and homogenized in a paint shaker (3 h). Four samples of 0.50, 1.00, 2.00 and 3.00 g are transferred to 125 mL flasks for digestion with nitric-sulfuric-perchloric acids as above. Instrumentation: Perkin-Elmer F-AAS with 3-slot burner, air (23 L/min)-acetylene (4.2 L/min) flame, λ = 232 nm; 16-20 mA. Detection limit (signal 2 x baseline noise) 1.7 μg/L MIBK, linear over the range 0.04 to a 4.0 μg Ni (standard solutions), within- and between-batch imprecision ≈ 10%. Cd and Au are potential interferents.

Faeces (Hassler et al., 1983) - Duplicate 4 g samples are dried in crucibles (15 h, 110-115°C), then dry-ashed in a muffle oven (2 x 12.5 h, 445°C), according to (Kjellström et al., 1978). The ash is dissolved overnight in 15 mL 1 M HNO_3 for analysis by flame AAS. Detection limit 0.16 μg Ni/mL of acid solution, corresponding to 0.7 to 1.6 mg/kg faeces. Within-batch RSD 4%.

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largely be circumvented by direct Zeeman-corrected GF-AAS determination of diluted or deproteinized samples, giving the analyst much better control over contamination during the pre-analytical phase.

F-AAS

In F-AAS, characteristic concentrations of 40 to 160 μg/L and detection limits of 2 to 10 μg/L (Tsalev, 1984) for Ni render the technique obsolete for the analysis of biological fluids. Extraction or pre-concentration are required when urine or serum are to be analyzed, and the abundant opportunities that these provide for sample contamination are best avoided by direct GF-AAS analysis. However, the flame may be useful for selected tissues or biological materials where Ni concentrations in the mg/kg range are expected. Some examples are given in Table 4. In general, a lean air-acetylene flame and high-solids burner give the best results, and high concentrations of acid and salt may cause interferences. Background correction is frequently required.

GF-AAS

Graphite furnace AAS with Zeeman background correction is the method of choice for most biological applications. Major advances in recent years are responsible for this situation. They include greater instrument sensitivity, the implementation of Zeeman background correction, and furnaces that provide for a rapid temperature rise during atomization (Stoeppler, 1984; Sunderman Jr. et al., 1984). Both pyrolytically-coated graphite tubes and the stabilized temperature platform furnace can be applied. Rothery reports a characteristic mass for Ni of 4.8 pg in Ar, using the Varian SpectrAA 30 instrumentation with a pyrolytically-coated, partitioned graphite tube and nitric acid matrix (Rothery, 1988), while Slavin and colleagues at Perkin-Elmer Corp. (Slavin et al., 1983) have used a platform and $Mg(NO_3)_2$ modifier to achieve a detection limit of 10 pg at a characteristic mass of 13 pg. In an argon atmosphere, $Ni(NO_3)_2$ is reduced to NiO at 650°C (Pupyshev and Nagdeev, 1979), which is further reduced to metallic Ni and vaporized with an appearance temperature of 1390±25°C (Akman et al., 1980). A maximum recommended ashing temperature of 900°C can be increased if modifiers such as $Mg(NO_3)_2$, or Pd with ascorbate are used. Carbide formation occurs, and so the use of pyrolytic coating is helpful (Tsalev, 1984). Nixon et al., using coated tubes and a serum matrix, found that the peak area subsequently achieved for Ni remained constant with charring temperatures up to 1600°C, while the background decreased to 0.10 A·s at this temperature and could easily be handled by Zeeman correction (Nixon et al., 1989). Three consecutive injections with intervening charring steps of 1500°C caused no measurable loss of Ni (Nixon et al., 1989). Rapid heating (maximum power) during the atomization step is necessary to decrease the atomization temperature to about 2500°C from 2900°C, and to minimize matrix effects. A cleaning step at higher temperature is often included after atomization. A typical selection of furnace programs recently used for the analysis of Ni in serum is given in Table 5.

Sunderman et al. (1988) have described three general sample treatments for preparing biological materials for Ni analysis by GF-AAS. (i) For tissues, food, faeces, etc. decomposition is necessary. Wet samples are minced and homogenized (1 g in 5 mL water) in plastic bags; dry samples, including bone, are minced. Acid digestion is accomplished by heating in capped tubes with 3:1:1 (v:v:v) concentrated nitric:sulphuric:perchloric acids (1 mL acid mixture + 1 mL homogenized sample) in an aluminium-block heater, sequentially at 110°C (30 min), 140°C (60 min), 190°C (30 min) and 300°C (60 min). Levels of Ni in lung, thyroid, adrenal, kidney, heart, brain, liver and pancreas have been measured by Zeeman-corrected GF-AAS after processing in this way, and were in the range of 10 to 370 μg/kg dry wt. (Rezuke et al., 1987). (ii) Serum, whole blood, saliva, etc. are analyzed after protein precipitation. While mixing on a vortex mixer, samples are acidified by addition of small amounts of concentrated nitric acid, then held at 70°C for 5 min. Protein-free supernatants are analyzed after centrifugation. (iii) Urine samples are measured directly after acidification with an equal volume of 0.1 M nitric acid. Selected procedures for Ni determination in biological samples by GF-AAS are given in Table 6.

TABLE 5

SOME RECENT FURNACE PROGRAMS FOR THE DETERMINATION OF Ni IN SERUM BY GF-AAS

From (i) Sunderman Jr. et al. (1984), (ii) Andersen et al. (1986), (iii) Bro et al. (1988) and (iv) Nixon et al. (1989).

	T(°C)				Ramp(s)				Hold(s)				Gas
	(i)	(ii)	(iii)	(iv)	(i)	(ii)	(iii)	(iv)	(i)	(ii)	(iii)	(iv)	
Dry*	100	100	100	—	1	40	10	—	1	20	80	—	on
	140	—	140	120	60	—	60	1	10	—	10	10	on
	190	200	190	160	30	60	30	70	5	10	5	5	on
Char/ash	—	—	—	700	—	—	—	30	—	—	—	10	on
	1200	1250	1100	1500	80	30	80	10	50	20	50	25	on[‡]
Atomize	2600	—	2500	—	0	—	0	—	5	—	5	—	off[‡]
	—	2700	2700	2750	—	0	1	0	—	6	3	5	off
Clean	2700	2800	—	2800	1	1	—	1	3	2	—	2	on

[*] These steps are repeated with a second sample introduction in study (iv).

[‡] In study (i), the gas flow is not turned off but is reduced from 300 mL/min to 30 mL/min for the last 5 s at 1200°C and for the atomization step.

TABLE 6

SELECTED PROCEDURES FOR Ni DETERMINATION IN BIOLOGICAL SAMPLES BY GF-AAS

Furnace programs are specified with ramp and hold times in seconds, with the shorthand (rx, hy) indicating a ramp to the specified temperature over x seconds followed by a hold time of y seconds at that temperature. A ramp time of 0 s indicates maximum heating rate of the furnace. An outline of the sample processing, analytical conditions and performance are provided when available; authors do not give a consistent level of detail.

Tissues (Sunderman Jr. et al., 1985) - Samples cut with a plastic serrated cafeteria knife are digested with a nitric:sulphuric:perchloric acid mixture as described in the text until clear and colourless and the final volume is about 50 μL. Samples are cooled and diluted to 4 mL with dilute HCl (conc. HCl diluted 1:9 v:v with water). 0.2 mL conc. HNO_3 is added. Instrumentation: Perkin-Elmer HGA-500 AAS with Zeeman correction, lamp current 25 mA, spectral band width 0.2 nm, λ = 232.0 nm, 50 μL sample injection. Furnace program: dry 100°C (r1, h1), 140°C (r60, h10), 190°C (r30, h5), char 1200°C (r80, h50), atomize 2600°C (r0, h5), clean 2700°C (r1,h3). Argon flow 300 mL/min, decreased to 30 mL/min during the atomization step and last 5 s of the char step. Detection limit 10 μg/kg dry wt. Imprecision (RSD): within-batch 15% at 76 Ni/g, accurate at 50 μg Ni/g in gelatin. Recovery of 200 ng added Ni 101 ± 8% (mean ± s).

Lung (Raithel et al., 1987) - To 1 g tissue in 40 mL long neck quartz vial add 3 mL conc. HNO_3 and 0.5 mL H_2SO_4. Heat to 120°C for 2 h. Increase temperature at half-hour intervals to 160°C, 220°C and 270°C. Dissolve residue in 10 mL water. Measure by method of standard additions. Instrumentation: Perkin-Elmer Zeeman 3030 ET-AAS, pyrolytically coated graphite tubes. Furnace program: dry 90°C (r10, h10), 110°C (r15, h10), char 1100°C (r15, h15), 1100°C (r1, h2; gas off), atomize 2600°C (r1, h3). Detection limit 0.8 mg/kg wet wt. Imprecision (RSD): within-batch 17% at 24 μg Ni/g, 0.5% at 60 μg/g; between-batch 16% at 24 μg/g. Recovery of 50 mg/kg spike 85%.

Urine (White and Boran, 1989) - Urine (0.5 mL) is diluted in sample cup with 1.0 mL 0.6% HNO_3 and mixed. Matrix-matched Ni standards (10 to 80 μg/L) are prepared in pooled urine of unexposed individuals. Instrumentation: Perkin-Elmer-500 ET-AAS with deuterium arc correction, pyrolytically coated graphite tubes, lamp current 25 mA, spectral band width 0.2 nm, λ = 232.0 nm, 25 μL sample injection. Furnace program: dry 120°C (r20, h20), char 1100°C (r70, h40), atomize 2500°C (r0, h5), clean 2700°C (r0, h5), clean 2700°C (r1, h4). Argon flow 300 mL/min, decreased to 30 mL/min during the atomization step. Linear across calibration range, detection limit 0.7 μg/L (2.1 μg/L in undiluted urine). Imprecision (RSD): within-batch 5%, between-batch 10.5%. Adequate interlaboratory comparison and agreement with Seronorm urine and Lanonorm 2 reference materials.

TABLE 6 (continued)

Urine (Sunderman Jr. et al., 1986) - Aliquots of urine are saved for specific density and creatinine determinations and the remainder is acidified with 1 mL HNO_3/100 mL urine. 1.0 mL acidified urine is transferred to a polystyrene centrifuge tube and diluted with 1.0 mL 0.1 M HNO_3. If turbid, centrifuge and decant into sample cups. Aqueous Ni standards of 4 to 80 μg/L are used. Instrumentation: Perkin-Elmer 5000 ET-AAS with Zeeman correction and peak integration, pyrolytically coated graphite tubes, lamp current 25 mA, spectral band width 0.2 nm, λ = 232.0 nm, 20 μL sample injection. Furnace program: dry 100°C (r1, h1), 140°C (r60, h10), 190°C (r30, h5), char 1200°C (r80, h50), atomize 2600°C (r0, h5), clean 2700°C (r1, h3). Argon flow 300 mL/min, decreased to 30 mL/min during the atomization step and last 5 s of the char step. Detection limit (blank + 3σ) 0.45 μg/L. Imprecision (RSD): within-batch 4.7% at 2 μg Ni/L, between-batch 5.8% at 16 μg Ni/L. No significant interferences were observed from added As, Ba, Bi, Cd, Cr, Co, Cu, Au, Fe, Pb, Mn, Hg, V, Zn (50 μM), Ca or Mg (25 mM).

Serum (Nixon et al., 1989) - Collect blood from a stainless steel syringe discarding the first 3 mL. All labware, pipette tips, etc. must be acid washed; manipulations are carried out at a class 100 clean-air station. 1.5 mL serum is mixed with 0.5 mL diluent (1% m/m Triton X-100 in 10^{-3} M HNO_3). Matrix-matched blank and standards of 2 to 8 μg Ni/L are prepared by addition of 0.5 mL Ni solution in same diluent to 1.5 mL pooled serum. Instrumentation: Perkin-Elmer 3030 ET-AAS with HGA-600 furnace, with Zeeman correction and peak integration, pyrolytically coated graphite tubes, spectral band width 0.2 nm, λ = 232.0 nm, 2 x 50 μL sample injection (100 μL total). Furnace program: dry 120°C (r1, h10), 160°C (r70, h5), 700°C (r30, h0), cool and repeat for a second injection, char 1500°C (r10, h25), atomize 2750°C (r0, h5), clean 2800°C (r1, h2). Argon flow 300 mL/min, off during atomization. Detection limit (blank + 3 S.D.) 0.06 μg/L. Imprecision (RSD): within-batch 3.2% at 1 μg Ni/L. Excellent agreement with Seronorm, NIST RM 8149 and the second generation reference material of Versieck et al. (Versieck et al., 1988).

Serum and whole blood (Sunderman Jr. et al., 1984; Sunderman Jr. et al., 1988) - Blood is collected through a polyethene cannula. Protein precipitation is carried out as described in the text, with addition of 50 μL HNO_3 to 1.0 mL vortexing serum of 200 μL HNO_3 to a mixture of 1.0 mL heparinized blood with 0.9 mL water. After warming and centrifugation, the protein-free supernatants are transferred to sample cups. Instrumentation: Perkin-Elmer 5000 ET-AAS with Zeeman correction and peak integration, pyrolytically coated graphite tubes, lamp current 25 mA, spectral band width 0.2 nm, λ = 232.0 nm, 50 μL sample injection. Furnace program: dry 100° C (r1, h1), 140°C (r60, h10), 190°C (r30, h5), char 1200°C (r80, h50), atomize 2600°C (r0, h5), clean 2700°C (r1, h3). Argon flow 300 mL/min, decreased to 30 mL/min during the atomization step and last 5 s of the char step. Detection limit (95% probability) 0.05 μg/L in serum, 0.10 μg/L in blood. Imprecision (RSD): within-batch 3.4% at 4 μg/L in serum and 3.8% at 4.7 μg/L in blood, between-batch 8.1% at 3.5 μg/L in serum. Recoveries: 96.9 ± 2.7% for 8 μg/L added to serum, 103.0 ± 5.5% for addition to blood.

Voltammetry

Due to the irreversible nature of the reduction of Ni(II) to Ni, standard anodic stripping procedures are not applicable to the analysis of Ni (Flora and Nieboer, 1980). The low solubility of Ni in mercury presents a further limitation to polarographic methods (Pihlar et al., 1981). Nickel chelates, such as that formed with dimethylglyoxime (DMG), improve the sensitivity of polarographic approaches by allowing the realization of an adsorption layer at the hanging mercury drop working electrode. In the case of $Ni(DMG)_2$, the enhancement is about 15-fold (Flora and Nieboer, 1980). Adsorption is allowed to proceed for several minutes and then the potential is swept, resulting in irreversible reduction of the Ni from the adsorbed chelate. Reduction begins at about -0.9 V (SCE). Use of a hanging mercury drop electrode maximizes adsorptive surface.

This approach to the determination of Ni in foods, beverages, blood, urine and biological reference materials was explored by Pihlar et al. (1981). 0.1 to 1 g of material in a long-neck Kjeldahl flask were digested with 0.2 mL conc. H_2SO_4 and 0.5 to 1 mL conc. HNO_3 by heating to 150°C. After digestion was completed by additions of 0.5 mL portions of 30% H_2O_2, peroxide was expelled by heating to the point of thermal decomposition of sulfuric acid. The sample was transferred to a 10 mL volumetric flask, the pH was raised to 9.2 with 0.1 M ammonium chloride buffer, and DMG (recrystallized from dioxane) was added to 1×10^{-4} M. After making to volume, the sample was deaerated for 20 min with a nitrogen stream and analyzed with standard additions. A PAR 170/174 Polarographic Analyzer was used with a Metrohm cell (EA 875-20), a hanging mercury drop working electrode and a counter electrode of Pt wire. A reference electrode of Ag/AgCl was connected to the analyte by a salt bridge filled with the supporting electrolyte (0.1 M ammonium chloride buffer). After adsorption (2 to 10 min at -0.7 V (SCE)), the potential was swept to -1.5 V at 20 mV/s. Determination of orchard leaves (found 1.30±0.07 mg/kg; certified 1.3±0.2 mg/kg), and values for Ni in several foods and beverages, urine (2.62 µg/L), blood (4.00 µg/L) and beard hair (10.6 mg/kg) were reported. Large amounts of Co may interfere, because the $Co(DMG)_2$ peak is only 150 mV more negative that that of $Ni(DMG)_2$. Normal pulse mode gives a higher peak current but a strongly sloping baseline: differential pulse mode has therefore been recommended for greater sensitivity. Subsequently, Ostapczuk et al. used a similar approach with a PAR 384 potentiostat, differential pulse mode (50 mV pulses, 57 ms duration, 0.5 s intervals) and subtraction of a sweep of the blank supporting electrolyte to determine Ni in blood, urine, saliva, liver and nail tissue (Ostapczuk et al., 1983). The implementation of faster electronics and square wave voltammetry has given a significant improvement in the detection limits of the technique, to about 2 ng/L (Stoeppler, 1984). However, it is unlikely that the methods needed for processing biological samples for determination by this technique can achieve blank values in this range.

TABLE 7

SELECTED PROCEDURES FOR Ni DETERMINATION IN BIOLOGICAL SAMPLES BY ICP-AES

Liver and kidney (Subramanian and Méranger, 1982) - 1 g samples are digested with 8 mL conc. HNO_3 + 2 mL conc. $HClO_4$ in 50 mL Pyrex beakers on a sand bath, taken to near dryness, dissolved in 1 mL conc. HCl, made up to 10 mL with 0.5 M HCl and pumped at 2 to 2.5 mL/min into a cross-flow nebulizer equipped with a Scott spray chamber. Instrumentation: ARL model QA-137 ICP-AES spectrometer, argon plasma, 27.12 MHz. λ = 231.60 nm, forward power 1600 ± 50 W, observation height 16 mm above the induction coil. Detection limit (blank + 2 S.D.) 0.01 mg/L. Correction for Fe and Mg required.

Bone (Mahanti and Barnes, 1983) - Powdered bone is dried at 100°C for 2 h and 1 to 3 g samples are weighed in 100 mL PTFE beakers. 15 mL conc. HNO_3 is added and slowly heated, then 30% H_2O_2 is added dropwise until dissolution is complete. The volume is made to 50 mL with water, 120 mL of 0.2 M EDTA is added, and the pH is adjusted to 5. The solution is passed through a column of 80 mg of poly(dithiocarbamate) resin, then the resin is digested with H_2O_2/HNO_3 and made to 5 mL with dilute HNO_3 for analysis. Instrumentation: Plasma-Therm model HFS-5000D argon plasma operating at 40.68 MHz. Sample introduction by electrothermal vaporization [100°C (10 s), 200°C (10 s), ramp at 800°C/s to 2100°C (3 s)]. λ = 341.45 nm, forward power 500 W, observation height 11.5 - 16.5 mm above the induction coil. Detection limit (without preconcentration 12 μg/L. 5.4 g/L Ca gives an interference equivalent concentration of Ni of 60 μg/L; background equivalent level, 44 μg/L; lowest quantitatively determinable concentration (5 x detection limit with 3 g sample dissolved in 50 mL), 0.1 mg/kg. With resin preconcentration, recovery of 5 μg Ni from human bone was 96% and analysis of IAEA H-5 animal bone gave 0.60 ± 0.01 mg/kg (expected value 1.51 ± 0.76 mg/kg).

Biological standard reference materials (Jones et al., 1982) - 1 to 3 g of dry sample are added to 25 mL conc. HNO_3 + 5 mL conc. $HClO_4$ in a 100 mL Kjeldahl flask and boiled 5 min past the end of perchlorate reaction (caution!). Following concentration on iminodiacetate chelating resin (Chelex 100; Bio-Rad, Richmond, CA) samples are made to 15 mL in 10% v:v HNO_3. Instrumentation: Jarrell-Ash model 975 argon ICP operating at 27.12 MHz, λ = 231.6 nm with background correction, 1100 W forward power, plasma flow 18 L/min, observation height 16 mm above the load coil. 0.15 mg/kg Ni said to be required for determination. Good results were obtained with several standard reference materials of oyster tissue and plant matter having Ni concentrations in the mg/kg range.

Milk (Camara Rica and Kirkbright, 1982) - 0.10 g freeze dried human milk solids are dissolved in 1 mL tetramethylammonium hydroxide (25% w:w aqueous solution) and made to 2 mL with water. After evaporating to dryness, the residue is dissolved in 2 mL water and 10 μL are transferred to a graphite rod in a Perkin-Elmer HGA-70 furnace. After drying (100°C, 30 s), ashing (900°C, 40 s) and atomization (2800°C, 1 s), the vapour is carried by an argon stream to a Plasma-Therm 27.1 MHz argon ICP interfaced to a 1200 lines/mm grating monochromator. λ = 341.6 nm, standard addition calibration and background correction used. Interferences from Cu, Mn and Mg. A reported detection limit of 2 pg seems unrealistic.

TABLE 7 (continued)

Subsequently the same group used acid digestion, liquid-liquid extraction with dithizone, $\lambda = 352.45$ nm, forward power 850 W and a viewing height of 33 mm above the load coil. They reported a detection limit (blank + 2σ) of 14 pg and a RSD of 2% at 0.21 mg/kg (Barnett et al., 1983).

Urine (Matusiewicz and Barnes, 1985) - NIOSH-NBS freeze dried urine is reconstituted in water. 50 μL samples are determined. Instrumentation: Plasma-Therm model 5000D ICP-AES spectrometer, Instrumentation Laboratory FASTAC II pneumatic nebulizer/aerosol delivery system to deliver sample to a model IL655 furnace for graphite furnace vaporization at 2500°C. Argon plasma, 40.68 MHz, $\lambda = 231.60$ nm, pyrolytically coated graphite tube with platform. Detection limit 0.9 μg/L (45 pg Ni) by peak area, 12 μg/L (600 pg Ni) by peak height. Urine reference material: found 1.05 mg/L (RSD 2.1%), expected 1.01 ± 0.11 mg/L.

Inductively coupled plasma-atomic emission spectrometry (ICP-AES)

In samples with relatively high concentrations of Ni (mg/kg or 10 ng/mL range), ICP-AES may offer the analyst a multielement capability that includes the reliable analysis of Ni. It may therefore be a useful adjunct to more sensitive methods (Maessen, 1987). The 231.604 nm line of Ni(II) is generally the preferred wavelength, with estimated detection limits of 15 μg/L (Winge et al., 1985). Iron and Mg are potential interferents. Fisher and Lee found interferences at this wavelength from Fe and Mn in faeces, with tolerable ratios (μg/L Ni:mg/L interferent not requiring correction) of 0.05 and 4.0, respectively (Fisher and Lee, 1982). Jones et al. have compared the results of several different digestion protocols and the use of iminodiacetate chelating resin on the determination of Ni in 10 different biological standard reference materials (Jones et al., 1982). They have concluded that good results can be obtained for Ni in the mg/kg range in these materials using nitric/perchloric acid digestion and pre-concentration; they provide an analytical scheme applicable to several trace elements (see Table 7). Recently Hofbauer et al. have used ICP-AES to determine a number of trace elements, including Ni, in renal stones (Hofbauer et al., 1991). Ni values from < 1 to > 100 mg/kg stone are reported, although very little analytical detail is given. Again, these detection limits generally restrict application of the technique to certain tissues and foodstuffs that have high Ni concentrations and do not present special analytical challenges. However, in such samples, the advantages of multielement analysis may be realized.

Miscellaneous methods

A number of other analytical approaches have seen limited (and not always successful) application to Ni analysis in biological matrices. Several of the more promising are described briefly here, in order to direct the reader to broader aspects of the relevant literature.

Neutron activation analysis shows poor sensitivity for Ni. Activation with thermal neu-trons followed by direct γ-spectrometry cannot be used because of the low cross sections of (n,γ) reactions of the Ni nuclides (Bem et al., 1983). Nevertheless, slices of autopsied, lyophilized lung from a welder were irradiated in sealed quartz ampoules, and Ni levels above a stated detection limit of 6 mg/kg were found in some slices (Kalliomäki et al., 1979). Radiochemical purification may be helpful, but has not generally been applied to Ni determination in biological materials (Versieck and Cornelis, 1989). Also, the main product (^{65}Ni; $t_{1/2}$ = 2.5 h) is a β^--emitter. Although it can be detected by Cerenkov counting, such an approach only allowed an estimate of less than 0.5 mg/kg in bovine liver (Pietra et al., 1975). In a different approach, Bem et al. used liquid scintillation counting to determine ^{65}Ni following neutron activation (Bem et al., 1983). Nickel was pre-concentrated prior to NAA by means of coprecipitation with α-benzildioxime, and other radionuclides were subsequently removed by two-phase isotopic exchange. Low bias determination of Ni in reference pine needles, orchard leaves and oyster tissue at the mg/kg level was achieved.

A micro-adaptation of energy dispersive X-ray fluorescence (EDXRF) has been used to compare the Ni content of normal and carcinomatous human breast tissue (Rizk and Sky-Peck, 1984). Surgical specimens were homogenized (200 g/L in water) in a Teflon homogenizer. Ten 5 μL aliquots of each specimen were layered onto Formvar films in a slide holder, air-dried under laminar flow, and placed in the vacuum chamber of the instrument at 200 μm of mercury. By X-irradiation for 500 s (18 mA, 35 kV) 16 elements were determined simultaneously. The Ni scattered peak was observed at 7 keV and Ni concentrations of about 1 mg/kg dry wt. were calculated after computer correction of the background continuum, as described (Sky-Peck and Joseph, 1981).

Mass spectrometric methods for analysis of Ni in biological fluids have been slow to develop. Nickel is approximately 91% ionized in a conventional argon ICP, and ICP-MS with quadrupole detection has a nominal detection limit of about 0.03 μg/L (Olivares, 1988). Unfortunately, polyatomic interferences from calcium oxides and hydroxides, as well as isobaric interferences from ^{58}Fe and ^{64}Zn, obscure the Ni masses. A multivariate regression enabled the determination of Ni at 70 μg/L in NIST freeze dried urine, and a detection limit of 1 μg/L in this matrix was estimated (Vaughan and Templeton, 1990). More recently, Xu et al. (1993) used this method to measure accurately Ni in a reference serum at 5 μg/L, and were able to measure Ni at this level directly in dilute urine after precipitation of calcium with oxalate. Perhaps more promising is the use of this approach for resolving isotopically enriched and naturally abundant Ni (Templeton and Vaughan, 1991; Vaughan and Templeton, 1990), thereby facilitating stable isotope tracer studies in human populations. Gas chromatography- mass spectrometry (GC-MS) has also been applied to the analysis of Ni in the NIST reference urine (Aggarawal et al., 1989). Samples were spiked with ^{62}Ni for isotope dilution analysis and evaporated to dryness for digestion with nitric acid. Volatile Ni chelates were then prepared by reaction with lithium bis(trifluoroethyl)dithiocarbamate for GC, and Ni determined by isotope dilution analysis of the MS signal. A sensitivity in the μg/L range was reported, although values about 15% above that recommended (70 μg/L) in the reference material were noted.

SPECIATION

Although other redox states occur transiently during enzyme catalysis, only Ni(II) is of significance as the stable form in biological systems. Therefore, speciation of Ni in biological fluids is directed toward identifying ligands and macromolecular binding components of Ni(II). It will be remembered that Ni-containing metalloenzymes have not been identified in higher animals, although the analyst of plant materials should note that acidification is insufficient to release Ni from precipitated urease (Sunderman Jr. et al., 1988). A number of high- and intermediate-molecular weight binding components of Ni have been fractionated in serum and cell cytosol, but not thoroughly characterized (van Soestbergen and Sunderman Jr., 1972; Sunderman Jr. et al., 1983; Templeton and Sarkar, 1985). This situation probably reflects the non-specific nature of ligands available for Ni in these fluids, although Ni does share specificity with Cu(II) for the N-terminal tripeptide binding site of albumin (Sarkar, 1983).

FUTURE PROSPECTS

The single most important improvement in biological Ni determination to be hoped for in the near future is the wider recognition of the need for rigorous contamination control. The co-evolution of analytical technology and pre-analytical control have made it apparent that many biological samples contain Ni at the sub-μg/L level. While methods such as GF-AAS and voltammetry can now claim low bias and precise performance at this level, wider implementation of clean-laboratory facilities is needed before much published misinformation can be replaced. It is hoped that isotope dilution-mass spectrometric methods, as have been helpful in determining V, Cr and Se in human serum (Versieck and Cornelis, 1989), will eventually provide a reference method for Ni. Careful studies of Ni speciation at the ultratrace level is a medium-term goal.

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Chapter 22

Selenium

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INTRODUCTION

The initial interest in selenium in medicine was caused by its potential toxicity, although the discovery of selenium as an essential element in human metabolism influenced explosively the need of selenium determinations in biological materials. In the determination of selenium in human materials it is necessary to take the various naturally occurring chemical forms of Se into consideration. When administered to humans or animals, the organic forms of the element are readily converted to various organo-selenium compounds. The available information suggest that the behaviour of selenium in man is quite similar to that in rats and other animals (Bopp et al., 1982)

Selenium is apparently metabolized in the erythrocytes and released back into plasma in a form which is protein bound. The identification in 1973 of glutathione peroxidase as a seleno-protein explained the essential function of selenium (Hoekstra, 1974).

Later studies have also identified trimethylselenonium ion as one of the urinary metabolites of selenium together with others (Nahaptian et al., 1984).

This chapter will focus on the determination of total selenium in clinical materials, especially body fluids such as whole blood, serum and urine. It is not the purpose to

present a comprehensive review of all the analytical methods capable of determining selenium, but rather an overview of various techniques with particular emphasis on those techniques used currently for both routine and research applications. There have been many literature reviews on selenium analytical methodologies in biological and environmental samples. Earlier reviews are those by Watkinson (1967), Olson et al. (1973), Alcino et al. (1973), Cooper (1974), Shrendrikara (1974) and Olson (1976).

Reviews since 1976 have tended to be limited to the determination of selenium in specific matrices (Crosby, 1977; Florence, 1982; Robberecht and Van Grieken, 1982; Rapits et al., 1983; Robberecht and Deelstra, 1984; Fishbein, 1984; Tölg, 1984; Lewis and Veillon, 1989; Tan and Rabenstein, 1989; Alt and Messerschmidt, 1988) and by specific analytical methodologies (Fishbein, 1984). Until fairly recently only total selenium has been determined even though selenium is known to occur in several inorganic and organic forms. Tan and Rabenstein (1989) discuss the analytical chemistry of organic and biochemical selenium covering glutathione peroxidase, selenoamino acids, and metabolites such as dimethyl- and dimetyldiselenide, trimethylselenonium and hydrogen selenide.

Even though a broad range of analytical methods are capable of determining selenium in human materials few of them satisfy clinical laboratory requirements of simplicity, reliability and speed, therefore we restrict this discussion to methods important in clinical chemistry.

Sample Preparation

Most of the methods for the determination of selenium in human materials require some sample preparation or pretreatment. The biotransformation of selenium in man, which is characterized by a step-wise biochemical reduction, leading to the binding to or direct incorporation of the element into proteins, apparently involves the formation of intermediate volatile species. Dimethyl selenide as well as many other organic forms of selenium and its halides are relatively volatile.

Most sample preparations for clinical materials when direct analytical methods are not applicatable involve the initial destruction of the organic constituents by mineral acids followed by the conversion of the element to the tetravalent state and subsequent determination by a variety of techniques. Extreme care and strictly controlled procedures are required to prevent losses of selenium (Gorsuch, 1959; Loyd et el., 1982). Preferred digestion mixtures involve combinations of nitric and perchloric (Nève et al., 1982) or nitric, sulfuric and perchloric (Watkinson, 1979; Welz et al., 1984) acids. The essential common factor for all methods is that reducing conditions are prevented and oxidizing conditions are maintained throughout.

In his reviews, Olson (1973 and 1976) covers most of the earlier, commonly used sample preparation procedures. More recently, Raptis et al. (1983) and Ihnat (1992) have discussed most sample digestion techniques, together with a comprehensive list of references. Reamer and Veillon (1981a) and Robberecht et al. (1981) reviewed the losses

of selenium from biological materials and May (1982) and Yläranta (1982) discussed losses of selenium by dry ashing.

Analytical Methodologies

A classification scheme for ranking the quality of a method has been proposed by the International Federation of Clinical Chemistry (Büttner et al., 1976). They define **definitive methods** as those that represent the ultimate in quality but are generally so sophisticated that they are not in common usage. Such methods for selenium are neutron activation analyses and mass spectrometry that employ isotope-dilution techniques negating the use of an external standard (Veillon and Alvarez, 1983).

Reference methods are those that have been tested against the definitive methods and/or represent the best method available for use under well controlled routine conditions. The fluorometric methods for plants (AOAC, 1970) and foods (Ihnat, 1974) developed by the Association of Official Analytical Chemists (AOAC) can be considered as reference methods when biological matrices are concerned.

The next category of methods are those routinely used but which may have known or unknown analytical biases or systematic errors depending on how well these methods have been studied and how much documentation there is in the literature. Such methods are the newer rapid techniques such as graphite furnace atomic absorption spectrometry and hydride generation atomic absorption spectrometry. It is probable that with sufficient documentation of accuracy and imprecision that one or both of these methods will be elevated to the status of reference methods.

Neutron Activation Analysis (NAA)

Neutron activation analysis (NAA) entails irradiation of the sample with neutrons, resulting in the production of radioactive Se isotopes whose concentrations are measured by monitoring γ-radiation of specific energies. Several types of activation have been employed for selenium determinations (Olson et al., 1973), however, the most widely used is activation with thermal neutrons. NAA can be used with or without destruction of the organic matter in the sample. The instrumental version in conjunction with γ-ray spectrometry (INAA) involves no chemical operations and is one of the very few techniques in the determination of selenium not requiring sample decomposition – a decided advantage. INAA is based on the measurement of both ^{77}Se (half-life 17 s) and ^{75}Se (half-life 120 days). With clinical materials few errors can be expected from effects due to thermal-neutron self-shielding or gamma-ray self-absorption. Biological samples generally contain high concentrations of sodium chloride which can cause a relatively large Compton background from ^{38}Cl and ^{24}Na. Should matrix activation lead to interferences or if β-activity is to be monitored, resort can be made to the radiochemical separation version (RNAA). Cornelis et al. (1982) discuss this and other potential problems in the determination of selenium in biological materials.

The isotope dilution approach of the technique (Veillon and Alvarez, 1983) could be considered **a definitive method**. Stable isotopes are non-radioactive and they can be used as metabolic tracers in all subjects including at risk groups such as pregnant females and infants where the use of radioisotopes is contraindicated (Sirichakwal et al., 1985; Janghorbani et al., 1982; Christensen et al., 1984; Kasper et al., 1984).

Because of its high sensitivity, the high specificity, the multielemental capability and simple sample pretreatment, neutron activation analysis is widely accepted as an excellent performer for the determination of Se in biological materials. Absolute detection limits have been reported of 10 ng Se for biological materials, imprecisions of less than 10% are routinely reported and with careful sample manipulation the imprecision may approach 1%.

NAA, however, has several important limitations in clinical analysis; one requires access to a nuclear reactor and extensive counting facilities, and the cost and time for analysis of samples are considerable.

Isotope Dilution Mass Spectrometry (ID-MS)

ID-MS is a sensitive and specific technique for determining elements which exist in at least two isotopic forms and for which a purified stable isotope preparation is available. A known quantity of this isotope is equilibrated with the sample which is then taken through processing to get the analyte in a form suitable for introduction into the mass spectrometer. The measurement of isotope signals at appropriate masses and calculation of ratios leads to concentrations of the original analyte. Spark-source mass spectrometry (SSMS) has been used by the U.S. National Institute of Standards and Technology to determine selenium in reference materials and solid samples (Olson et al., 1973; Veillon and Alvarez, 1983). The high volatility of selenium in a high temperature spark with subsequent low sensitivity and relatively poor precision and accuracy restricts the applicability of SSMS. Selenium is also not considered a good element for thermal ionization mass spectrometry (TIMS), the most precise and accurate of the current mass spectrometric techniques.

Inductively coupled plasma mass spectrometry (ICP-MS), a relatively novel technique with high sensitivities for most elements, presents major attributes not available with other methods of isotopic analysis; simple requirements for chemical processing, high sample throughput, acceptable level of measurement precision and bias, multi-element capability and reasonable accessibility and capital cost. The relatively low sensitivity for selenium, however, precludes the direct measurements in clinical matrices without analyte pre-concentration (Hieftje and Vickers, 1989). The present state of development of ICP-MS for application to clinical matrices indicates that the technique can provide selenium isotope ratio measurements with the required accuracy and precision (Ting and Janghorbani, 1987; Ting et al., 1989).

Combined gas chromatography/mass spectrometry has the advantage over other mass spectrometry techniques of shorter analysis time and less expensive equipment.

The lack of suitable chelating agents for some analytes of interest is the main limitation of GC/MS. However, Reamer and Veillon (1981b) have developed a suitable chelate and method for the determination of selenium in biological materials. This method has been further adapted to a double-isotope method for studies of selenium metabolism (Reamer and Veillon, 1983; Swanson et al., 1983a, 1983b; Moser-Veillon et al., 1992). Under routine conditions this method has proven to be well suited to determine total selenium in foods, diets, human breast milk, infant formulae, plasma and serum, red blood cells, feces and urine. The method has the accuracy expected for a definitive method, the precision is about 2% at the 100 μg Se/L level, and the absolute detection limit is about 50 pg.

Atomic Absorption Spectrometry (AAS)

The introduction of atomic absorption spectrometry in 1955 by Walsh has brought about a preferred analytical technique among clinical chemists in the field of element determinations. Flame atomic absorption atomization techniques with solution aspiration is not sufficiently sensitive (detection limits varies from 0,05-3 mg Se/L for most clinical applications where sub-mg/L concentrations are encountered. The sensivity can, however, be improved by generation of volatile selenium hydride and subsequent atomization in argon-hydrogen flames or electrically heated quartz tubes. Electrothermal atomization of solutions in graphite tubes has developed rapidly since the analytical use was first proposed and studied by L'vov (1961). For most clinical chemists this technique may be the most appropriate technique to analyze samples for low concentrations of selenium.

Graphite Furnace Atomic Absorption Spectrometry (GF-AAS)

GF-AAS methods combine a very high atomization efficiency with a 100-1000 fold increase in sensitivity compared to the conventional flame technique. For this reason and also because of the capability of handling solid samples as well as samples which normally requires sample pretreatment, GF-AAS is one of the most popular methods in trace and ultratrace element determinations in clinical chemistry.

Various authors have described the application of this technique for selenium determinations in body fluids (Saeed et al., 1979; Alfthan and Kumpulainen, 1982; Brown et al., 1982). As selenium is subject to losses during the drying and charring steps, matrix modification techniques are recommended. Addition of metal ions to the sample is beneficial in that selenium compounds are thermally stabilized. Studies using radioactive tracers have shown that while a number of metal reagents prevents the volatilization of **inorganic** occurring selenium at low charring temperatures (Alexander et al., 1980) only nickel, silver and partly molybdenum have substantial effects on metabolized forms of selenium present in whole blood, serum and urine and allow for charring up to 1200°C (Saeed et al., 1979; Alexander et al., 1980).

Aside from the extensively studied volatility interferences, it has been demonstrated that the conventional method of background correction which is based on the use of a continuum source (D_2), is subject to spectral interferences from iron and for phosphate decomposition products (presumably PO and P_2) (Saeed and Thomassen, 1981). Even though these spectral interferences are highly reduced by matrix modification with either nickel, a nickel/platinum or a nickel/palladium matrix modifier, the use of a Zeeman based instrument is highly recommended (Bauslaugh et al., 1984; Radziuk and Thomassen, 1992; Hoenig, 1991).

Using pyrolytic graphite tubes, platform atomization and integrated absorbance readings a **Zeeman-based** instrument provides an acceptable situation for selenium determinations and permits most analyses to be performed against matrix matched calibration graphs. In Table 1 recommended procedures for the direct GF-AAS determination of selenium in body fluids are listed.

TABLE 1

SUGGESTED PROCEDURE FOR THE DETERMINATION OF SEE IN BODY FLUIDS

Sample preparation

Dilute serum and whole blood 1:5 with 0,2% Ni matrix modifier containing 0,2% (v/v) Triton X-100.

Standardization

Prepare appropriate standards using animal/human whole blood and serum containing low levels of selenium.

Instrumental Parameters:

Wavelength 196.0 nm   Signal mode: Peak area
Spectral Band Width 2.0 nm
Electrodeless Discharge Lamp.
Zeeman background correction. Continuum source (D_2) may be applied for plasma/ serum matrices

Furnace Parameters (dependent of the system)

Drying steps: 100-250°C
Charring steps: 800-1100°C
Atomization step: 2500°C Max Power Mode
Gas flow: Interrupted
Cleaning and cooling steps
Pyrolytic graphite tubes. Wall atomization.
The direct GF-AAS-determination of selenium in urine is still under debate (Hoenig, 1991). Acid digestion is therefore recommended prior to hydride generation AAS or fluorometric determinations.

For samples other than serum and whole blood some form of sample pretreatment and/or digestion is necessary. GF-AAS offers a very low detection limit (20-50 pg), imprecisions that range from 1-5% and semiautomated instrumentation.

Hydride Generation Atomic Absorption Spectrometry (HG-AAS)

Atomic absorption spectrometry with hydride generation offers the advantages of excellent sensitivity and simple instrumentation for the determination of hydride forming elements, e.g. selenium, in a wide variety of matrices. The determination of selenium by HG-AAS has been reviewed by Verlinden et al. (1981). The most common approach is to liberate H_2Se by using $NaBH_4$ as a reductant, and strip the generated volatile hydride from solution with argon or nitrogen into a heated quartz atomization cell. In spite of many reports that the bias of this method is very dependent on the decomposition technique used, Welz and co-workers (Welz et al., 1984; Welz et al., 1987) have in a IUPAC-project shown that this technique can have a low bias. Their recommended decomposition procedure with nitric, sulphuric and perchloric acids at a final temperature of 310°C gave results on human body fluids that agreed very well with those obtained by other techniques. No interferences were found from other constituents of human body fluids under the conditions used. The HG-AAS technique has been shown to have absolute detection limits ranging from 0.1 ng to 60 ng. Imprecision varies according to care and expertise of the user. Weltz et al. (1984) reported a RSD of 2.6% and a routine detection limit of 5 μg/L of selenium on 0.5 mL sample aliquots of serum, plasma, whole blood, erythrocytes or urine. This detection limit may be lowered to <1 μg/L by applying larger sampler aliquots or larger amounts of the digestion solution for the determination. Flow injection is an efficient approach for introducing and processing liquid samples in HGAAS. The smaller sample size, higher sample throughput, better tolerance to chemical interferences, improved absolute limits of detection, lower consumption of reagents and ease of automation of flow injection has further strengthened HG-AAS as a reliable methods for selenium determinations in body fluids (Burguera, 1989; McLaughlin et al., 1990).

Fluorometry

The fluorometric method is a popular technique and is one of the methods of longest standing in the determination of selenium in biological materials.

Interlaboratory - collaborative testing with foodstuffs (Ihnat, 1979) plant materials (Horwitz, 1980) and human body fluids (Kumpulainen and Koivistoinen, 1981; Ihnat et al., 1986a, 1986b; Nève et al., 1992) has demonstrated excellent performance leading to its acceptance e.g. by the AOAC as an official method for the determination of selenium in foods and plants. The organic matrix of the sample is destroyed by an acid mixture such as nitric/perchloric or nitric/perchloric/sulphuric, the analyte complexed with 2,3-diaminonaphthalene (DAN) and quantitated by molecular fluorescence.

The reactions with 2,3-diamino compounds are specific for tetravalent selenium, but since sample preparations are normally carried out under oxidizing conditions which could

yield hexavalent selenium, it is essential to effect a reduction from hexavalent to the tetravalent state before complexing reaction. The most common method used is heating with hydrochloric acid (Watkinson, 1966). Exact instructions for apparatus, reagents and procedures are given by the AOAC (AOAC, 1970). The fluorometric assay has been successfully automated by Brown and Watkinson (1979) with a high throughput. As far as method imprecision is concerned the fluorometric method should at the 100 mg Se/kg level give a RSD of 5% (Ihnat et al., 1986a), the sensitivity is good and detection limits of 10-100 ng have been reported (Rapits et al., 1983; Ihnat, 1974; Anal. meth. com., 1979).

With close attention to details and the use of quality assurance programs, the fluorometric method is one of the simplest, least expensive and most versatile of all the methods under discussion.

Quality Assurance, Standard Reference Materials

Four recent interlaboratory cooperative studies revealed a broad scatter of selenium results in the analysis of human serum, whole blood and urine (Kumpulainen and Koivistoinen, 1981; Ihnat et al., 1986a, 1986b; Nève et al., 1992). The main objective of the IUPAC studies which were directed under the auspices of the IUPAC Clinical Chemistry Division, Commission on Toxicology, was to establish control materials for total selenium determinations and to assess the performance of divergent analytical methods applied to the analysis of normal levels of selenium. A number of skilled laboratories throughout the world applied their preferred routine or research methodologies to the determination of selenium in lyophilized human serum, whole blood and urine samples; isotope dilution mass spectrometry, graphite furnace atomic absorption spectrometry with and without sample pretreatment, hydride generation atomic absorption spectrometry, fluorometry, instrumental and radiochemical neutron activation analyses, x-ray fluorescence spectrometry and proton-induced X-ray emission. For this population of laboratories the determinations based on graphite furnace atomic absorption spectrometry, hydride generation atomic absorption spectrometry and proton-induced x-ray emission exhibited the largest systematic differences among laboratories.

For any analytical method, a quality assurance program must be developed to help ensure the traceability and precision of the results. Once a method has been established, the single most effective way of maintaining the quality of the results obtained is by regular use of well characterized, **suitable** quality control materials which are treated in an identical manner to the samples. Ideally, the quality control materials should have a matrix and analyte concentration similar to the samples and more than one material with different levels of analyte should be used.

Numerous Se projects were set up by both analytical and medical communities - unfortunately in many instances without elaborate quality control measures. Although a great many investigations have been reported on research into and application of a variety of analytical methods for the estimation of Se in human tissues and fluids, very few biological and human materials are available with established concentrations of Se for

method verification and data quality assurance. Biological reference materials prepared in the 1960's and 1970's by individual scientists such as Bowen (1967) and by organizations such as the National Bureau of Standards and the International Atomic Energy Agency are currently available. Recently **human body fluids** such as serum, whole blood and urine have been introduced as certified or proposed quality control materials in the determination of Se. See chapter on Reference Materials for Trace Element Analysis, Table 3 for currently available reference materials for Se.

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Chapter 23

Thallium

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INTRODUCTION

In the past, reviews of thallium have been, either from a mainly toxicological (Zitko, 1975), environmental (Schoer, 1982; Ewers, 1988), or analytical point of view (Sager and Tölg, 1984; Sager, 1986; Griepink et al., 1988; Sager, 1992). Here a combination of these three aspects is given. The technological important uses of thallium and its compounds remain few, and the summaries given in the reviews quoted above are still valid, are not given here.

Occurrence and geochemical cycling

Properties of thallium and its compounds

There are two stable isotopes: 203-Tl (29.52% nat. occurrence) and 205-Tl (70.48% nat. occurrence). Thallium can occur in the zero-, mono- and trivalent state. The element is easily oxidized and of no pronounced technical use.

Thallium(I)
Inorganic and organic compounds of Tl(I) are easily soluble in water. Among the least soluble are (Seidell, 1958; Selig, 1980):
0.1 - 1 g/L: TlBr, TlJO$_3$, Tl$_2$S, salts of fatty acids
0.01 - 0.1 g/L: Tl$_2$CrO$_4$, TlJ
0.0005 g/L :Tl-tetraphenylborate.
Under physiological conditions, as well for trace analytical procedures, no pre-cipitations are considered; coprecipitations, however, can be used for analytical separations (Sager, 1986; Sager, 1992). In the system Tl-S, 8 different phases are known besides mixed sulfides with other metals (Fialkov and Muzyka, 1952). Tl$_2$S can add elemental sulfur as well as polysulfide, which lowers its solubility. In open air, on the other hand, Tl$_2$S is easily oxidized to soluble Tl$_2$SO$_4$. Monovalent thallium forms stable com-

plexes with sulfur-containing ligands, some of which can be evaluated analytically (with dithizone, dithiocarbamates). Towards oxygen-containing ligands, cyanide and fluoride, however, it acts like an alkali-metal (Sillén and Martell, 1964).

Thallium(III)

Thallium(III) is a strong oxidizing agent with capabilities of complex formation and protolyzation ($[Tl^{3+}][OH^-]^3 = 6.3 \cdot 10^{-46}$) (Seidell, 1958). The compounds of trivalent thallium are easily soluble. It oxidizes many organic ligands. As the $[TlCl_4]^-$ and $[TlBr_4]^-$ complex, it forms stable ion association with quaternary ammonium compounds or other ligands with basic oxygen or phosphorus (Sager, 1986).

Alkylthallium compounds

Compounds of the type R_2TlX and $RTlX_2$ (X = monovalent anion) are generally more stable than the respective Pb-compounds. R_3Tl immediately protolyzes to yield R_2Tl^+ + OH^- + RH (Huber et al., 1978). They are formed in anaerobic sediments by biomethylating bacteria.

Thermal properties

Thallium is moderately volatile in elemental form, as well as in halide, oxide or nitrate forms. Inorganic compounds of Tl(III) thermally decompose to yield the respective Tl(I)-compounds, and the organic ones pyrolyze to $Tl^°$ or Tl_2O (see Table 1).

When heating the samples, the volatility of Tl-compounds has to be taken into consideration. Either oxygen or hydrogen, respectively hydrogen diluted with nitrogen, can be used as carrier gas. The volatility in oxide, chloride or elemental forms leads to evaporation of Tl and some other trace elements selectively from the solid sample, which has to be considered also in technical processing of thallium-containing ores and other materials.

TABLE 1

THERMAL PROPERTIES OF INORGANIC THALLIUM COMPOUNDS

	MP °C	BP °C
Tl-element	303	1457
$TlNO_3$	206	430
TlF	327	655
TlCl	430	720
TlBr	480	815
Tl_2O	596	1080 (Cubicciotti, 1970)
Tl_2O_3	–	875 (in O_2; Cubicciotti and Keneshea, 1967)

TABLE 2

NORMAL POTENTIALS (Bellavance and Miller, 1975)

$Tl^+ e = Tl$	$E° = -0.3363$ V
$Tl^{3+} + 2e = Tl^+$	$E° = +1.25$ V
$Tl(OH)_3 + 2e = TlOH + 2 OH^-$	$E° = -0.05$ V

TABLE 3

POLAROGRAPHIC HALF-WAVE POTENTIAL IN 0.1M-KCl, E = -0.43 V (versus saturated calomel electrode)

Complexes of $(CH_3)_2Tl^+$, type ML (Bugarin et al., 1988; Bugarin et al., 1989)

Glycine	1.19
L-Cysteine	3.62
N-acetyl-L-cysteine	2.62
DL-penicillamine	3.81

ENVIRONMENTAL AND TOXICOLOGICAL SIGNIFICANCE

Background levels of occurrence

The level of soluble thallium present in the sea (e.g. Pacific Ocean, Atlantic Ocean, Irish Sea, Australian Coast) is between 9 and 16 ng/L (Matthews and Riley, 1970). This is remarkably lower than in fresh waters. In natural sea water (pH 8.1), the oxygen content is sufficient to oxidize Tl(I) to Tl(III), because formation of chloro-complexes stabilizes the trivalent state. In the Pacific Ocean, 80% of the thallium was found to occur as Tl(III), and only 20% as the sum of Tl(I) and alkylthallium compounds (Batley and Florence, 1975). As Tl(III) is easily adsorbed and coprecipitated, it continuously moves down to the sediments.

In the HNO_3 extract of Austrian soils above the crystalline in the Bohemian massive, a mean of 0.5 mg/kg was found. Above sedimentary clays and gravels from the tertiary between the Alps and the Danube, a medium value of 0.2 mg/kg was found, and in the Northern limestone Alpine region, a somehow broadened distribution with a mean of 0.36 mg/kg (Hofer et al., 1990).

Range of occurrence of thallium (nonanthropogenically polluted areas)

	Concentration in mg/kg
Granite	0.35 - 3.60 (Siedner, 1968)
	2.7- 4.0 (Butler, 1962)
	1.1- 1.4 (Dupuy et al., 1973)
Basalt	0.021-0.058 (Heinrichs, 1979)
Limestone	<0.005-0.19 (Gorbauch et al., 1984)
Gypsum	0.04 - 0.13
Iron ores	0.006- 1.9
Sediment (Pacific Ocean):	0.27 - 1.25 (Dvoretskaya and Bojko, 1979)
Deep sea nodules	87 - 118 (Kirchner et al., 1980)
	23 - 226 (Iskowitz et al., 1982)
Coals:	<0.1 - 3.3 (Sager, 1993)
Coal slags and ashes	<0.1 - 2.4 (Sager, 1993)
Fuel oil	0.02- 0.10 (Gorbauch et al., 1984)
Soils (Germany)	0.17- 0.53 (Eschnauer et al., 1984)
Soils (China)	0.29- 1.17 (Wenqi et al., 1992)
Fertilizers	0.02- 0.57 (Eschnauer et al., 1984)
Plants for animal feeding	0.02- 0.08 (Scholl, 1980)
Vegetables (edible parts)	0.03- 0.20
Red wines	0.09- 0.48 (Eschnauer et al., 1984)
White wines	0.04- 0.3
Milk	0.010-0.033 (Scholl, 1980)

Reference materials from the German Environmental Specimen Bank

Mussels II	0.248 (Waidmann et al., 1984)
Alga II	0.0272
Earthworm	0.0203
Alga I	0.0143
Poplar leaves	0.0076
Pig liver	0.0029

Contamination and environmental mobilities

Atmosphere

Combustion of coal as well as dust from zinc- and cadmium processing industrial plants are the main sources of thallium emission into the atmosphere (Magorian et al., 1974). Thallium is accumulated together with other volatile species in filter and flue dust; there it is remarkably soluble in water.

Fresh water ecosystems

Sources of thallium input are effluents or drainage waters from landfills from Zn-, Pb- or Cu- smelters, as well as from sulfuric acid and pigment production. Because of the low

need for thallium, recovery from deposited landfills was not attempted for a long time (Magorian et al., 1974).

Contaminated sediments

In contaminated soils and sediments, thallium is largely mobile. Thallium is readily adsorbed by freshwater sediments, but quantitatively remobilized again with neutral ammonium acetate or neutral ammonium chloride (Sager, 1991). In the stream sediment of the Lenne near a smelter, Tl at a total of 2 mg/kg was found to be very immobile. Only 15% was dissolved by 1M HNO_3, and less than 1% at pH > 2 (Günther et al., 1987), thus indicating the input of non-processed ore material.

Contaminated soils

As Tl is volatile both under reducing and oxidizing conditions, as chloride, nitrate, oxide or element, it is enriched in the flue dust of smelters, cement plants, etc. Even in the surroundings of brickworks, an up to 5-fold increase of thallium contents of soil and grass was detected (Brumsack, 1977). Similarly, Tl from coal is volatilized during combustion processes; crude oil contains lower Tl amounts. At a contaminated site in Germany with more than 50% clay content (mainly illite), the main fractions of thallium were exchangeable versus ammonium acetate at pH 7, and residual. The contamination with thallium, as well as the exchangeable proportion, strongly increased with decreasing grain size, thus demonstrating the effect of active surfaces (Lehn and Schoer, 1987). Data about the selective detection of dimethyl thallium in environmental samples have not been available yet. Thallium uptake by green plants, above all rape (Brassica napus), correlated with the exchangeable soil fraction, minor amounts were assumed to be transferred to the plant from the hydroxylamine reducible fraction (Lehn and Schoer, 1987).

Transfer to green plants

Thallium is strongly accumulated by mature rape leafs (brassica napus), and to a lesser extent by lettuce and cabbage, but not by turnips, zucchini, maize, oats and grass.

BIOTRANSFORMATION IN ANIMALS AND MAN

Ingestion and distribution in the body

Analyses of human body parts after ingestion of lethal doses of Tl are scarce. In a young man from India who committed suicide, most elevated levels were found in liver and brain, but not in the kidneys (Tewari et al., 1975).

If Tl is applied intravenously, it is rapidly absorbed by tissue cells. Within a few minutes only 39% remains in the plasma. Maximum concentrations in muscles are reached after 4-8 h, and finally in the brain after 24 h (Rauws, 1974).

Thallium also crosses the human placenta, but the human fetus is not as seriously affected as the mother (Formigli et al., 1985).

Recently, Tl-201 scintigraphy is used to get a picture of metabolic dynamics, which can be interpreted in terms of the metabolism of thallium itself.

In myocardial and muscle tissue, thallium rapidly moves into the tissues (experiments with dogs and rabbits, see above), and the uptake is increased by stress. The distribution of thallium in the myocardium is a marker of myocardial viability, because abnormal regions show significantly less thallium uptake than normal ones. In coronary scintigraphy using the γ-radiating isotope 201-Tl, a first portion of tracer is injected at stress, followed by reinjection at rest after some hours. Myocardial regions that demonstrated low differential uptake were located in an area supplied by a totally occluded coronary artery. Regions with reduced activity are regarded as reversibly ischemic if thallium activity significantly increases after 24 h, and irreversibly abnormal if it does not increase (Dilsizian and Bonow, 1992).

Effects on animal and man

Effects on animals

In natural waters, Tl is not precipitated as a carbonate nor as an hydroxide, and is also hardly bound to soluble humic material. Therefore, toxicity is largely independent of water characteristics, such as hardness, dissolved organic carbon, or suspended matter (Nehring, 1962; Zitko et al., 1975).

Acute toxicities of Zn and Tl, as well as of Cu and Tl, are not additive (Zitko, 1975). Toxicity effects can vary widely with age. Zooplankton, like daphnia, react more sensitively than the fish which feed on them. Fresh water fish exert toxicity symptoms after 1-2 days.

In mammals, the main effects of intoxications are in the nervous and digestive systems, accompanied by renal necrosis and loss of hair. Toxic effects of thallium to rats have been thoroughly investigated, on the one hand, as a model for humans, and on the other, because thallous sulfate has been used as poison against rats for a long period of time (Friberg et al., 1979).

For guinea pigs, dogs and men, single doses of 15 mg/kg body weight (b.w.) are totally lethal (Zitko, 1975; Righetti and Moeschlin, 1971) without therapeutical treatment. 50% lethality is obtained by single doses of 15 mg/kg b.w. for rats, and 16-27 mg/kg b.w. for mice (Krasovskii et al., 1980).

In rats, single doses of 50 ng/kg body weight already provoke symptoms, whereas 5 ng/kg b.w. have no effect. Morphological changes occur in kidneys, liver, and epithelial cells of the intestine. Single doses of lethal amounts fed to rats leads to death of the animals within 24 - 54 h. Most amounts remains in the stomach which develops lesions, while kidneys and adrenals gain significantly more weight, because of swelling of tubulus cells. Subacute poisoning of rats with 4 times 1 mg/kg b.w. leads to loss of hair and diarrhoea within 96 h, and to similar weight increase of kidneys. Tl was readily adsorbed and quickly distributed throughout the whole body, but there were large interindividual variations. In blood, Tl was found slightly more within the erythrocytes than in the plasma (Leloux et al., 1987). A further important target organ for thallium are the testicles,

whereas it does not move to the skeleton (Krasovskii et al., 1977). After i.v. injection into rats, thallium plasma concentration declined at 5 min and 196 min half-time, moving to the tissues but not to the kidneys (Henning and Forth, 1981). 40 h after a single dose application, the Tl-contents of kidneys, testicles, brain and cerebellum still increase, whereas in the other organs they are already declining. The relative distribution between the various organs is dose dependent (Sabbioni et al., 1980).

In rats, extensive placental transfer results in a similar time course of Tl-levels in maternal and fetal organs. In the brain, however, Tl removal was faster in the fetus than in the mother, because of less retention by the immature organism (Formigli et al., 1985). In cats, at doses of 4 mg/kg b.w., hypotony and ataxy occur, which are mainly due to pathological changes of primary sensoric neurons. Further, dogs and cats which fed on Tl-containing rodenticides developed hemorrhagic gastroenteritis together with hepatic and renal damage. If they survive longer, further symptoms develop such as loss of fur, bloody lesions of the skin, tremor and paralysis of muscles (Friberg, 1979). In mice suffering from chronic thallium intoxication, degenerative changes in the female genital tract were observed (Formigli et al., 1985). In dogs, Tl-induced testicular atrophy has been described, with simultaneous premature release of germinal cells into the seminiferous tubule (Formigli et al., 1985).

The dynamics of thallium in living tissues can be observed by scintigraphy using the γ-radiating isotope 201-Tl. In dog hearts, 2 min after an injection pulse into the supplying blood, there is a nearly linear relation beween percent maximal retention in the contracting cells and myocardial blood flow. Significant back diffusion occurs 20 min afterwards, thus lowering tissue retention by the increased blood flow (Melon et al., 1992). Similarly, in rabbit hearts, maximum tissue retention was obtained after 1 min. At low flow, a pulsed dose of thallium escaped completely after 10 min. At high flow, tissue retention was much higher, followed by rapid exponential decline for the first 5 min, and a slow decline for more than 1 h (Marshall et al., 1991).

Effect on humans

To elucidate metabolic and toxic effects of thallium, many experiments with model animals (see above) have been done. The results, however, are not fully compatible to human, because of differences in food composition (e.g. Na/K-proportion).

Toxicity symptoms

Besides accidental or suicidal ingestion, there is occupational exposure of some groups of employees in the production and processing of heavy metal ores, manufacturing and use of thallium, its alloys and compounds, and in certain cement factories from roasting pyrites and ingestion of dust from the electric filter (Schaller et al., 1980). Whereas the excretion level in urine from persons without known occupational contact with thallium was found to be lower than 1.1 mg Tl/kg creatinine, it significantly increases upon exposure. No influence of the age of the person examined and the duration of employment, as well as no influence of alcohol and nicotine consumption was noted.

Therefore, concentration of thallium in the urine can be considered a suitable parameter for the assessment of the presence of thallium in the body, without detection of further symptoms (Schaller et al., 1980).

Normal levels in man

To establish relationships between element concentrations and toxic effects in the general population, as well as in occupationally exposed workers, profound knowledge of baseline data for all tissues of paramount importance is essential. Recently, Minoia et al. (1990) gave reference data for urine, blood and serum from about 400 healthy Italian subjects. Data from smokers, pregnant women, sportsmen, persons suffering from cardiovascular disorders, mental diseases or cancer, were discarded. They coincide with older data from Schaller et al. (1980); Weinig and Zink (1967) and Hamilton et al. (1972), see Table 4. Smokers, as well as vegetarians, excrete significantly more thallium than non-smokers (Weinig and Zink, 1967).

TABLE 4

REFERENCE VALUES FOR HEALTHY HUMAN SUBJECTS

	Mean	Range
Urine	$0.42 \pm 0.09\,\mu g/L$	0.07-$0.7\,\mu g/L$ (Minoia et al., 1990)
Blood	0.39 ± 0.05	0.15-0.63
Serum	0.18 ± 0.009	0.02-0.34
Urine		0.3-1.1 mg/kg creatinine (Schaller et al., 1980)

ANALYTICAL METHODS FOR BIOLOGICAL SPECIMENS

As thallium usually occurs in the $\mu g/kg$ and low mg/kg range, the analytical procedure must achieve sufficient sensitivity, and possibly include enrichment steps. Only a few multielemental techniques, such as the old optical emission spectrography, proton induced X-ray emission, or mass spectrometric techniques yield reliable data on the trace levels usually encountered. In most cases, there has to be a proper investigation for thallium, possibly leaving some of its environmental impacts unknown.

Less common analytical methods, or those not suitable for the economical analysis of thallium in biological matrices are omitted for reasons of space. These include atomic fluorescence, solid-sampling atomic absorption, X-ray fluorescence, gas chromatography, and ion-sensitive electrodes. They have been reviewed intensively, e.g. by Sager (1986), and by Griepink et al. (1988).

Recently, much progress has been made in mass spectrometric techniques (especially ICP-MS), and first attempts in speciation studies. Atomic spectrometric and electrochemical determination methods are being developed in terms of application to real matrices.

Adsorption

In general, working with dilute solutions requires attention to adsorption at the vessel walls. From weak acid solution, Tl^+ adsorption was found to increase in the order polyethene–polypropene–glass–paraffin–rubber (Morgan et al., 1967). At borosilicate surfaces, at pH < 4, no losses occurred at the 1 mg/L level, whereas at pH > 10, there was appreciable adsorption (Smith, 1973).

Sample decomposition methods

Wet oxidation with mineral acids

The general aim of sample decomposition is the destruction of a representative amount of sample to yield a homogeneous solution, sufficiently mineralized to be suitable for subsequent determination, preferably for multielemental analysis. In the case of thallium, the problem is rather to dissolve a sufficient amount of material to reach the limit of determination than to cope with problems of blanks.

As thallium is moderately volatile as an halogenide, oxide or nitrate from nearly any kind of matrix (Sager, 1984), dry-ashing procedures of large sample weights may often lead to severe losses (Scholl, 1980). Similarly, fuming with HF in open vessels leads to volatilization losses above 140°C, and with HBr above 160° (Rechenberg, 1982). For biological matrices, usual methods of wet decomposition with acid mixtures can be applied, like reflux with aqua regia (author's experience), HNO_3/H_2O_2 (Scholl, 1980), or H_2O_2/HCl (Haas and Krivan 1984). Charring with $HNO_3/HClO_4$, which is usually the most effective wet-oxidation open method, has to be critically checked, however.

To reduce the amount of acid required, the fallout from the laboratory atmosphere, and to enable much more rigorous digestion reactions at higher temperatures than possible at the boiling points of the respective acids, the digestion of micro amounts can be done in pressure bombs. The material used for the proper vessel inside a massive steel jacket is quartz, PTFE or glassy carbon (Šinko and Gomišček, 1972; Kotz et al., 1972; Kotz et al., 1979). Typically, 0.1 g serum or 50 mg milk powder have been mineralized in the smallest size of pressure vessels (10 mL), or 0.5 g serum /150 mg milk powder in 25 mL vessels (Šinko and Gomišček, 1972; author's experience). For biological materials, the sample weight is limited because of the pressure evolved from the reaction products, most of which are gaseous: each carbon goes to CO_2 and simultaneously HNO_3 goes to NO or NO_2. Degradation of biological material under pressure at 180°C maximum in PTFE bombs completely mineralizes the organic matrix with a few exceptions. Phenylalanine gives e.g. nitrobenzoic acids, histidine gives imidazole-4-caboxylic acid, and tryptophan a mixture of aromatic compounds (Würfels et al., 1989). Using glassy carbon, up to 230 °C can be reached; the problem is to keep the vessel tight (Kotz et al., 1979). As oxidizing

agents, HNO_3, or HNO_3/HF are sufficient, even for coals (Sager, 1993). Coal samples are very resistant and need at least 14h at 140°C in the pressure bomb (Sager, 1993); higher temperatures to coal samples cannot be applied because too much pressure develops inside the vessel. Aqua regia is not suitable, because it yields additional pressure from NOCl.

To overcome the long heating and cooling periods of the steel jackets of the bombs, and problems with their corrosion products, microwaves (2450 MHz) have been used to heat mineral acid mixtures inside tightly screwed PTFE- or polypropylene vessels. The absorption of microwave energy for $H_2O:HNO_3:HF:H_2SO_4$ is approximately 100:80:57:45. In the use of microwaves, the control of temperature and pressure inside the vessel is critical, and can not be easily estimated from outside; some curves are given by Kingston and Jassie (1986). For example, 5 mL of HNO_3 reach 140°C within 3 min at 150 W (1/4 of full power). For decomposition, HNO_3, HCl, HF, H_2SO_4, and KOH have been tested (Kingston and Jassie, 1986; Buresch et al., 1987). Aqua regia is not suitable because of additional pressure from NOCl. $HClO_4$ or H_2O_2 can cause explosions (author's experience) and are thus preferably avoided. Microwave energy and reaction time for complete destruction of the sample without damaging the bombs have to be optimized for each matrix.

For practical routine analysis, it is not easy to homogenize each material sufficiently to obtain representative micro-samples. Slightly larger sample weights can be treated if a prereaction period between the sample and the oxidizing acid in the open succeeds in the evolution of much gaseous products, and the bombs are closed at elevated temperature.

As an alternative to wet oxidation of organics, low temperature ashing in an oxygen plasma is possible, the ash can be dissolved in nitric acid (Waidmann et al., 1984). For subsequent determination by AAS, enzymatic solubilisation of tissues in alkaline solutions is sufficient, e.g. by the proteolytic enzyme subtilisin in the presence of trizma base (2-amino-2-hydroxymethyl propane (1,3)diol) at 55°C (Carpenter, 1981).

Special combined decomposition/separation techniques

From rocks and soils, Tl can be quantitatively volatilized in a stream of hydrogen (Geilmann and Neeb, 1959) as well as in a stream of oxygen (Heinrichs, 1979). In case of organic materials, volatilization of tarlike substances into the apparature makes the technique far from ideal, therefore the authors used ashed samples. On dry ashing, however, some Tl might already be lost (Scholl, 1980). Tl can be volatilized from dried biological samples after admixture with silicic acid, by combustion in oxygen in a special quartz apparatus (Trace-O-Mat-R). Volatile traces are condensed on a cool-finger and dissolved with nitric acid or hydrochloric acid. Many matrix elements remain in the slag on the sample holder. Compared with conventional tube furnaces, dead volume and blanks are much lower (Han et al., 1982; Liem et al., 1984).

Separation methods

If the final determination is subject to interference by concomitant elements, or the detection limits are insufficient for the samples to be analyzed, separation methods are necessary, increasing the time of analysis per sample, and thus the costs. As low amounts of thallium have to be expected on the one hand, and many determination methods suffer from interferences on the other, separation steps have to be considered sometimes. According to the author's experience, losses because of incomplete recovery of thallium are more probable than the introduction of blanks.

The average blank, leached with water into new 10 mL serum tubes, was determined (by ICP-MS) to be 4 ng for EDTA containing tubes, 2 ng for silanized tubes, and not detectable for empty tubes (Paudyn et al., 1989).

Recently, the trend is towards final determination methods of sufficient sensitivity to avoid methods of separation and enrichment. Ion exchange and chromatographic methods require much time, besides complete destruction of the organic matrix, which limits their application in the analysis of biological materials, and thus are not given here. They might attract future attention, however, in speciation studies.

Coprecipitation

Trace constituents can hardly be precipitated into individual compounds because the amounts are usually too low to exceed the solubility product of the respective solid. They can be sorbed, however, at a solid collector precipitated from the sample solution, leading to a uniform solid matrix. The success of the separation can be easily estimated by non-skilled workers by watching the properties of the precipitate, prior to measurement. Coprecipitation can be done with a large number of samples simultaneously, with subsequent separation of the collector with the wanted traces by centrifugation. Coprecipitation reactions need not be at equilibrium. They depend on the collector employed, on accompanying ions, and time and temperature of the precipitation, which influence the surface of the newly formed precipitate. Thus, in the presence of strong buffer solutions, fatty acids or surfactants, coprecipitation behaviour might largely change. Complexation of the collector ions or adsorption of organics on the precipitate can lead to insufficient recovery of the wanted traces, and even prevent the desired precipitation. Coprecipitation methods have primarily been designed for the enrichment from large volumes of environmental water samples, as well as for the determination of impurities in metals, or heavy metals in salts and biological matrices.

In case of analysis of water and biological samples, coprecipitation is usually employed to separate Tl together with other trace elements from large amounts of water, as well as alkali and alkaline earths metals, and from halogenides. As solid collectors, $MnO_2.xH_2O$, $Fe(OH)_3$, $Al(OH)_3$, $Zr(OH)_4$ and $Mg(OH)_2$ have been tested in detail, and can be easily dissolved in nitric acid (Geilmann and Neeb, 1959; Kreshkov et al., 1975; Veselago and Novikov, 1977; Efremov and Stolyarov, 1959; Efremov and Alexeeva, 1959). As an example for variable conditions, collection of thallium from acetate buffer with $MnO_2.xH_2O$ is quantitative, but in the presence of excess nitric acid, hydroxylamine or oxalate, the

precipitate, if any, does not take thallium with it (Sager, 1992b). If Fe-hydroxide is pre-cipitated with ammonia instead of NaOH as is usual for coprecipitation, Ga, In and Tl are coprecipitated, but Cu, Zn, Cd, Ag, Hg and Cr(VI) are largely left in solution, which is important for the enrichment from sulfide ores (Yan et al., 1988).

Similarly, thallium is quantitatively sorbed on ZnS at pH 5.6, on Bi_2S_3 from dilute sulfuric acid, and on CuS, whereas CdS is only partially effective (Rudnev and Mazur, 1957; Rudnev et al., 1961).

Among common organic reagents, precipitation of dithiocarbamates from pH 4-7 has been used for a multielement collection of heavy metals including thallium (Berndt et al., 1981). Quite selective collectors for the enrichment of thallium traces from environmental waters, are AgCl, AgJ, and Hg_2Cl_2 precipitates, especially in the presence of EDTA (Morachevski et al., 1958; Jackwerth and Graffmann, 1968; Borovitskii et al., 1966; Berndt and Jackwerth, 1976 and 1977).

Selective sorption of Tl(I) from neutral solution has been achieved on mixed K-Co/Ni-iron (II)cyanides on silica gel support (Alikin et al., 1984).

Solvent extraction

Dissolved species are distributed between two immiscible liquid phases, usually an aqueous and an organic one. It is a rapid technique for a small series (up to 20), with low-cost equipment. Enrichment factors of 5 are easily achieved. It is well documented for thallium, because most of the publications dealing with separation methods employ this technique. Non-destructed organic material mainly also moves to the organic phase, which may cause problems in spectrophotometric or graphite furnace-AAS final determinations. It is largely lost, however, if the separated thallium is stripped into an aqueous layer again.

In case of a large number of samples, environmental regulations pertaining to the waste of the organic solvents used, e.g. halogenated hydrocarbons, benzene etc., have to be considered.

Solvent extraction of thallium(I)

Complexes of thallium(I) are weak in most cases. The selectivity can be increased by masking accompanying cations with cyanide, EDTA, or citrate. For the isolation of thallium together with other heavy metals, dithiocarbamate, dithizone, thiooxine or diantipyrylthiourea in weak alkaline media can be used (Keil, 1981; Ackermann and Angermann, 1970). Solvent extraction of Tl(I) with 2-mercaptobenzothiazole into chloro-form can be made quite selective in the presence of tartrate and cyanide at pH 6-9 (Itawi and Turel, 1984). Dimethylthallium (reacting rather like Tl^+, but nominally trivalent) is extractable, e.g. as PAN complex into $CHCl_3$ (Günther and Umland, 1989).

Thallium(III) halogenides and reagent solvents

It is not necessary to use special organic reagents for the separation of thallium. Anionic Tl(III)-complexes with halogenides or thiocyanate react with Lewis bases to form solvent extractable ternary complexes. As the Lewis bases, the solvents themselves can

be used, such as ethers (diisopropylether, dibutylether), esters (amylacetate), ketones (methylisobutylketone, cyclohexanone), as well as amines (trioctylamine, diphenylamine, benzylaniline). Tetrachlorothallate(III) quantitatively moves to diethyl-, diisopropyl- and dibutylether within the broad range of 0.8 - 10 M HCl (Irving and Rossotti, 1952). With regard to volatility and toxicity, diisopropylether is the solvent of choice, but in case of subsequent flame-AAS of the extract, methylisobutylketone yields the most sensitive signal (Elson and Albuquerque, 1983). Extraction of tetrabromothallate is even more selective. From 0.5 M HBr, only Au and some Sb, Sn and Hg are extracted together with Tl, but they can be stripped with hydroxylamine/0.5 M HBr (Sager and Tölg, 1982). Back-extraction into an aqueous phase can be done with 5% ascorbic acid (Cammann and Anderson, 1982; Günther and Umland, 1988), or ammoniacal ammonium sulfite (Sager, 1992b).

Thallium(III) halogenides extracted with reagents into inert solvents

From acid solution, ternary complexes of Tl(III) with halogenides and reagent bases are extracted together into rather inert solvents. Examples are the extraction from 0.2M HBr with tri-n-octylamine into benzene, from 0.001 M HCl with PAN into chloroform, with rhodamine B into benzene, and from 3-8 M HCl with dioctyl sulfoxide into benzene (see photometric determinations). Like in the case of reagent solvents, Au, Sb and Hg are difficult if not impossible to separate from Tl(III). From ammoniacal EDTA solution, dimethylthallium is extracted with PAN into $CHCl_3$ (Günther and Umland, 1989).

Final determination methods

ICP with atomic emission detection (ICP-AES)

The sample solution is aspirated into a toroidal shaped plasma, where atomization and excitation occurs. The emitted radiation is either measured simultaneously at previously selected atom lines, or one line after the other is sequentially scanned. The actual sensitivity depends on many parameters of the apparatus used, such as the type of nebulizer, optics, power, gas-flows, and observation height (for general review, see Broekaert and Tölg, 1987). If solvent extraction is applied as a technique for separation and pre-concentration, however, it should be kept in mind that the plasma is easily distorted and extinguished by organic solvents. For thallium, there is some choice between different lines, but sensitivity is rather poor in any case, so that the direct application to decomposition solutions of rocks, soils, and biological samples is limited.

At an excitation frequency of 50 MHz, detection limits are better than at 100 MHz (Boumans, 1989). As thallium is easily excitable, optimum signal to background ratios are obtained at low powers (Montaser et al., 1981). The most sensitive emission line in the ICP is probably at 190.864 nm (Barnes, 1980; Boumans, 1989), but vacuum conditions are needed.

The prominent line for atomic absorption, 276.787 nm, is frequently used for emission also. In pure 0.1% HNO_3, 0.12 mg/L have been achieved as detection limit on this line,

which is the worst among 19 elements studied (Peng et al., 1990). Sensitivity can be improved to 0.05 mg/L by thermospray vaporization, but effects of salts and buffer substances on the thermospray design were not investigated. Spectral interferences occur with more than 100-fold excess of Fe (276.75 nm). If Tl 276.787 nm is measured in the second order, there might be an overlap with Ba I 553.548 nm (Anderson and Parsons, 1984).

At 351.924 nm, spectral interferences have not been reported yet. Whereas the sensitivity has been found to be relatively low with regard to other Tl lines by Barnes (1980), others found the sensitivity in the second place (Dorado Lopez et al., 1982). 377.572 nm, which is often used in DC-arc-excitation methods, may not be resolved from Ca (377.59 nm), Ni (377.56 nm), Ti (377.61 nm), and V (377.62 nm, 377.57 nm, 377.52 nm) (Botto, 1981), and above all Ar at 377.545 nm (Anderson and Parsons, 1984). Therefore, no practical application of this line has been found in ICP-AES.

At 535.046 nm there are hardly spectral interferences, but the transparency of the optics may not be ideal in this spectral region for each device.

Matrix Na up to 0.5%, which is often encountered in biological matrices, causes a depression of the signal up to 10%, with considerable variations from day to day. At the cost of losing sensitivity, a zone of minimum interference could be located in the plasma. The overall optimization of parameters was not affected by a change from Meinhard to cross-flow nebulizer. Unfortunately, the wavelength used in this work is not given (Ebdon and Carpenter, 1988).

Mass spectrometry

Mass spectrometry is one of the most powerful multielemental determination methods, allowing the estimation of non-pollution levels in a multielement run. In field-desorption MS, 10 pg Tl could be detected in 2 μL of non-ashed sample solution (homogenized rat brain) by means of an isotope dilution technique (Schulten et al., 1978), offering possibilities of microlocal trace analysis. Ionization of $CHCl_3$ extracts from plant cytosols with Ar^+ (secondary ion MS) enabled the detection of dimethylthallium + directly in the mass spectrum (Günther and Umland, 1989).

If spark-source MS is used, biological samples have to ashed, preferably with high-frequency oxygen, because organic degradation products interfere with the interpretation of the ion spectra. The resulting ash is formed on an electrode together with graphite or carbon. Less bias can be achieved by spiking with 203-Tl, taking into account the natural isotope ratio 203-Tl/205-Tl = 3/7 (Hamilton et al., 1972), thus also controlling for possible losses in the ashing step.

Low ionization energy (6.07 eV) makes Tl an element readily suitable for thermal ionization from e.g. Re filament (ReO^+ is isobaric with Tl, but obviously this does not matter); ionization has already been achieved at 700°C, whereas neighbouring Pb needs higher temperatures (Trettenbach and Heumann, 1985; Heumann et al., 1981). Biological material and sewage sludge have been analyzed this way after wet ashing with HNO_3/HF or low-temperature ashing in O_2. The method was so sensitive that blanks of the proced-

ure, done in a clean room with ultrapure chemicals, could be determined as 0.05-0.3 ng Tl (Waidmann et al., 1984; Weinig and Zink, 1967; Waidmann et al., 1992).

By means of ionization with Ar^+ (secondary ion mass spectrometry), it is possible to determine dimethyl thallium beneath inorganic thallium, preferably after extraction from ammoniacal EDTA with PAN into $CHCl_3$. Identification is due to the molecular mass and the isotope ratio 203-Tl/205-Tl = 3/7 (Günther and Umland, 1989).

ICP with mass-spectrometric detection (ICP-MS)

This relatively new technique, which has gained application for some routine work during the last years, offers the advantage of lower detection limits than ICP-AES, simple spectra, and linear calibration of 5 orders of magnitude (Sansoni et al., 1988). The ion: atom ratio produced in the plasma greatly influences the height of the signal. To cope with ionization matrix effects, isotope dilution methods have been applied. During measurement, high vacuum has to be maintained in the detection system. Thallium is measured as 205-Tl, the 203-Tl isotope can be used as a spike for isotope dilution (Park and Hall, 1988). Easily ionizable elements lower the signal, but Tl is less affected than Y and Li (Gregoire, 1987). In natural waters from a granitic region, a detection limit of 10 ng/ml was achieved (Sansoni, 1988).

Sample introduction into the ICP by electrothermal vaporization leads to a dramatic improvement in analyte transport efficiency. Losses during the ashing stage are overcome by isotope dilution with 203-Tl (Park and Hall, 1988).

Atomic absorption spectrometry (AAS)

Flame methods

For thallium, determinations at the 276.78 nm line with an acetylene/air flame are used throughout. Matrix problems are very low, but the sensitivity with regard to the low level of occurrence is poor. The sensitivity can be increased by mounting a slotted quartz tube on the burner head STAT = "slotted tube atom trap") (Milner, 1983), which leads to a detection limit of about 20 mg/kg in the solid sample, which is insufficient for the analysis of biological matrices. In MIBK extracts, determination of thallium is much more sensitive in flame AAS than in aqueous solutions (till about 7-fold). This can be used for solvent extraction of thallium from 0.1M HBr (Hubert and Chao, 1985), as xanthate at pH 8 (Aihara and Kiboku, 1980), or as iodide with tri-n-octylphosphinoxide into MIBK, and direct aspiration of the organic phase into the flame.

Graphite furnace AAS

Atomic absorption determination in the graphite furnace is a single element method, but it can be automated and controlled.

General interferences on the signal

In pure solutions, charring can be done up to about 500°C, and atomization at about 2300°C, but the latter figure may vary due to the instrumentation used. Atomization from nitric or dilute sulfuric acid is preferable. In the presence of halogenides and perchlorate, the charring temperature as well as the signal obtained is less; matrix modifiers permit charring at higher temperatures (see below). The chloride interferences on Tl are in part caused by volatilization of TlCl in the pyrolysis stage, and in part by formation of TlCl in the gas phase during atomization. Low levels of halogenides can be volatilized from dilute sulfuric acid prior to charring of thallium (Fuller, 1975; Kujiray et al., 1979; Welcher et al., 1974; Slavin et al., 1982; Sauer and Eckhard, 1981; Hamid et al., 1990; Welz et al., 1988).

For thallium, the detection limits achieved in pure solutions at 276.8 nm are fairly good (about 0.3-0.5 μg/L); but in urine: 3.0 μg/L. Other cations in large excess, such as Fe and P, also depress the signal (Machata and Binder, 1973). Nitric acid soil extracts could be determined by platform atomization/standard addition without further separation (Hofer et al., 1990).

Tube material

Tl does not form carbides during the determination cycle. Coated tubes may even yield lower signals, depending on the matrix (Beaty et al., 1980). Coating with Ta and W or Zr greatly enhances possible charring temperatures (up to 1000°C), without change of the sensitivity (Hamid et al., 1990). Whether platform or wall atomization, or peak area/ peak height evaluation are more suitable, seems to depend on the actual apparatus and matrix. In a urine matrix, platform atomization was found superior because it improves the time resolution of analytical signal and the background. At high heating rates, the easily volatilizable thallium appeared at the beginning of the background signal, making evaluation via peak height preferable to avoid errors in background compensation (Berndt and Sopczak, 1987).

Matrix modifiers and background compensation

As no severe spectral overlaps are known, background correction with a deuterium lamp is usually sufficient if no additional matrix modifier is added. Deuterium is in many cases also sufficient if some sulfuric acid is added to displace HCl and HF during the drying stage, but not in matrix phosphate. After the decomposition of plants with $H_2SO_4/HNO_3/H_2O_2$, the slope of Tl in the resulting solution was only 3-25% concerning height, and 17-36% concerning area. Addition of Li nitrate, ammonium salts as well as phosphate were not successful (L'vov et al., 1978; Dulski and Bixler, 1977).

Addition of a matrix modifier allows a sufficient rise in the maximum allowable charring temperature to mineralize organics in the sample, and to displace halogenides. The aim is to reach uniform sensitivity in any sample, so that evaluation of the signals via a calibration curve is possible. Additional smoke from any of the modifiers, however, necessitates the use of Zeeman background compensation.

The Tl line at 276.8 nm exhibits an anomalous Zeeman pattern. The relative absorbance passes a maximum at 0.9 T field strength, and declines at higher field strengths (Fernandez et al., 1980).

1.5 g/L Pd + 1.0 g/L Mg in nitrate form, added as matrix modifier, permitted charring up to 1000°, and 2.0 g/L Pd even up to 1400°C, but 500 mg/L Ni in nitrate form had a low effect (charring <600°C). Thus, urine samples have been analyzed without preceding separation (Welz et al., 1988). For chloride medium in an organic solvent, addition of Pd as a matrix modifier also allowed much higher charring temperatures (700°C) from the tube wall, but additional ascorbic acid improved the signal only slightly (Collett and Jones, 1991). Atomization from a transversal heated platform with simultaneous Zeeman background correction compensated the strong background from a phosphate/Mg/Pd matrix modifier, which allowed charring up to 950°C (Huth and Schulze, 1991).

Combination with separation methods

To cope with matrix effects, and to achieve some enrichment, suitable separation methods can be applied, e.g. solvent extraction, coprecipitation, or volatilization directly from the inorganic solid sample (Heinrichs, 1979; Han et al., 1982; Sager, 1984). As the volatility of organic chelates is low, and the volatility of halogenides from organic solvents remains the same, organic phases obtained from solvent extractions have been put directly into the furnace. In this case, however, carry-over in the autosampler has to be prevented by an organic solvent, at least by an isopropanol/water mixture.

The performance of coated tubes may be very bad, when organic solvent extracts are used, like the extract of diethyldithiocarbamate in toluene (Kubasik and Volosin, 1973). In the latter case, nearly double the sensitivity was obtained by inserting a tantalum foil into the tube to avoid soaking the graphite with toluene. Drying of aqueous Pd matrix modifier solution on the tube wall prevented soaking of coated tubes with di-isopropylketone sample solution (Collett and Jones, 1991).

Back extraction into an aqueous phase, e.g. with ammoniacal ammonium sulphite, avoids problems with the autosampler, non-reproducible soaking of the tubes and smoke from non-decomposed lipids, and is thus preferable.

Electroanalytical determinations

In the most sensitive electroanalytical methods, exclusively treated within this context, the analyte ion is electrodeposited on an electrode from an electrically conducting sample solution. Current and potential of subsequent redissolution are due to the concentration and the kind of ion to be determined. For thallium, the reversible redox couple Tl^+/Tl° at about -0.5 V versus saturated calomel electrode is used (Bellavance and Miller, 1975). Infinite tolerance towards alkali, alkaline earths and halogenides are great merits for the analysis of biological materials. Because of the preconcentration step included, thallium determination *is more sensitive than atomic spectrometric methods.* For thallium, the multielement capabilities of the method can hardly be used, because lead and frequently cadmium have to be masked with excess of complexants, leaving just Tl in the potential

range of determination. As thallium complexes are rather weak, the electroanalytical thallium signal is nearly non-affected by complexants in solution. Complexes of more noble metals like Tl, however, can be shifted to the range of Tl deposition by complexation (Pribil, 1972; Roux et al., 1975). If in the stripping the potential of a reversible redox couple present in the solution is passed, background current is much enhanced (Kryger, 1980; Liem et al., 1984). Surfactants provoke shifts of the signal, which can be used for electrochemical masking (see below); residual organics from incomplete digestion, however, can yield ghost peaks, like in the case of coal and rubber (example given by Gorbauch et al., 1984). Combustion techniques avoid this problem.

Non-decomposed organics may cause background currents which harm the determination. Serum digests obtained with $HNO_3/HClO_4$ in pressure bombs had to be fumed with H_2SO_4 (Sinko and Gomiscek, 1972), and digests and aqua regia extracts from soils had to be fumed with H_2O_2 prior to analysis (Lukaszewski and Zembrzuski, 1991). For calibration, standard addition has to be used throughout.

Hanging Hg drop electrode

Tl forms an amalgam with Hg, which is stable within a broad concentration range. Tl^+ is deposited about 1.6 times faster than Tl^{3+}, therefore starting with monovalent Tl and standard addition is preferable. Aliphatic and aromatic Tl compounds can be also reduced at the hanging mercury drop electrode; contrary to Tl^+, the peak potential is strongly pH dependent. At pH 6 (citrate), the peak of dimethylthallium appears separately from inorganic thallium, but diphenylthallium coincides (Dhaneshwar and Zarapkar, 1980; Hoeflich et al., 1983).

In dilute HCl, there are overlaps of Tl with Pb, and partially with Cd and Sn. To avoid co-deposition of Sn, Cd and partially Pb, the deposition potential has to be kept as low as possible, at minimum -0.8 V (versus saturated calomel) in dilute HCl (Neeb and Kiehnast, 1968). Fe(III) has to be reduced with hydroxylamine (Gemmer-Čolos et al., 1989). EDTA, citrate or tartrate as conducting electrolyte are well suited to complex interferents.

Overlap from neighbouring Pb and Cd can be avoided by suitable choice of the supporting electrolyte, like citrate, tartrate or EDTA in about neutral medium. In citrate/EDTA at pH 7, a 10,000-fold excess Pb does not interfere with the Tl peak, but the sensitivity is slightly reduced. The influence of surfactants on the thallium signal is low, whereas other peaks, which are close together in dilute mineral acid, are shifted apart. For matrix Bi and Pb, tetrabutylammoniumchloride addition to 0.2 M EDTA pH 4.5 is suitable (Ciszewski and Lukaszewski, 1983; Ciszewski, 1985). For determination of Tl in matrix Cu, addition of surfactants containing an ethylene oxide chain completely suppresses the peak of Cu in 0.2 M EDTA at pH 4.5 (Ciszewski and Lukaszewski, 1983). The mechanism of masking the Pb and Cd signals towards Tl by dodecylsulfate in presence of citrate, tartrate and EDTA seems to be a decrease in the charge transfer reaction (Opydo, 1992). Excess Fe can be electrochemically masked by Rokafenol N-3 in an oxalate supporting electrolyte (Lukaszewski et al., 1987).

After extraction of the dithiocarbamates into benzene from a neutral solution, anodic stripping voltammetry has been performed in the organic layer by addition of methanol

and $NaClO_4$ as supporting electrolyte. There is nearly no difference between peaks from Tl dithiocarbamate and inorganic Tl; 1,000-fold excess of In and Cd did not interfere (Labuda and Vaničkova, 1987).

Mercury thin film electrode

By electrolytic deposition of Hg at pH < 4 on an inert electrode, a layer of metallic mercury is generated, serving as a new electrode surface for subsequent use. Compared with the hanging mercury drop electrode, in the absence of complexants and surfactants Tl and Pb are resolved, but Cd coincides (Roux et al., 1975).

Urine, and saliva could be directly polarographed for thallium after addition of a EDTA/acetate supporting electrolyte (pH 4.7) on a mercury-film graphite electrode (Kauffmann et al., 1984), with a detection limit of 0.01 μg/L for 15 min deposition time (far above background levels).

Spectrophotometric and fluorimetric methods

Simple colour reactions of thallium or alkylthallium with PAR, PAN or similar reagents are neither sensitive nor selective, and can be applied only as general post-column colour reactions for ion-chromatography (Yan et al., 1988).

Determinations via ternary complexes between Tl(III) - Cl/Br/SCN - cationic dye can be made quite selective, because of preceding separation, and sensitive, because of the possibility to choose a very strong absorbing dye. The ternary complex has nearly the same spectrum as the dye, but it is separable from the excess dye by extraction into an organic solvent from acid solution. Cross interferences are usually similar, whether the dye is measured by photometry or fluorimetry. If preceding enrichment by volatilization, or at least solvent extraction, is done, environmental background levels can be reached with simple equipment. Prior to the determination, Tl has to be oxidized. Chlorine, bromine, Ce(IV), peroxydisulfate are suitable in the cold.

In combination with a suitable liquid-liquid extraction method, with regard to the normal range of occurrence of possible inorganic interferents, the selectivity of the photometric determination is excellent. Severe interferences, however, are caused by incompletely destructed organics (from humics, chlorophyl etc.), which carry carboxylic groups forming extractable ion associates with the cationic dye. Before contacting the dye, the excess oxidants as well as non destructed organics have to be destroyed, e.g. by fuming with H_2SO_4.

Thus, for decomposition of soil samples etc., combustion, volatilization, or at least pressure decomposition methods are preferable prior to a photometric method. Among inorganics, the main positive interferences derive from Au, Hg, Sb and Ga; coextraction of tungstate, chromate, borate, perchlorate, thiocyanate and iodide are less frequently reported. Larger excess of fluoride depresses the signal. Among the dyes, brilliant green, methyl violet, crystal violet, methyl green, rhodamine B, safranin T etc. have been used (see e.g. Marczenko et al., 1974; Miketukova and Kohliček, 1964; Scholl, 1980;

Gregorowicz et al., 1981; Mikaelyan et al., 1989). A complete survey of selectivities and interferences would go beyond the scope of this work.

High selectivity could e.g. be achieved by oxidation with Ce(IV), extraction from 0.5 M HBr into diisopropylether, cleaning the extract with hydroxylamine/HBr, and finally shaking with acid rhodamine B dye solution. Only an aliquot of the dye with respect to $TlBr_4^-$ moves to the organic layer and can be measured at 555 nm. As the absolute detection limit was 40 ng Tl, enrichment from 10 g of biological sample is necessary to reach the level of occurrence in biological matrices (Sager and Tölg, 1982).

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Chapter 24

Vanadium

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ENVIRONMENTAL AND TOXICOLOGICAL SIGNIFICANCE

Industrial uses

Vanadium has an abundance in the earth's crust of about 0.2% (Clark, 1975). It is quite evenly distributed in minerals. A few commercial deposits contain more than 3% vanadium pentoxide, but normal concentrations are 0.1 – 1% (NAS, 1974). The main sources of vanadium are vanadium sulphide (patronite), carnotite and titanomagnetite ores. Many crude oils contain considerable amounts (even about 0.1%) of vanadium, notably those from Venezuela. The ash obtained from burning vanadium-containing oils may have many tens of per cent vanadium. Vanadium can be extracted from fuel ashes. A considerable amount of vanadium production is based on the extraction of converter slag in the steel industry, which can contain 2 – 10% (Michels, 1973) or even 25% (NAS 1974) vanadium pentoxide. Vanadium is usually manufactured by converting the vanadium minerals to a water- soluble form (Levanto, 1969).

Vanadium and its compounds are widely used in industry in the following processes (Alessio et al., 1988):
— in the production of special vanadium-iron steels used for the manufacture of high speed mechanical tools;
— with other metals (Cu, Cr, Co, Ti) in the composition of corrosion and temperature resistant alloys, e.g., in the aeronautical industry;
— in iron and steel refining and tempering;
— in hard metal production;
— in the manufacture of pigments, printing inks and paints;
— in the form of V_2O_5 as a catalyst in the pharmaceutical industry.

The main vanadium exposure risks at the workplace occur during the processes of vanadium extraction and preparation from the minerals and to a greater extent during cleaning and repair of petrol containers and oil-fired boilers. For the biological monitoring

of an occupational vanadium exposure the determination of urinary vanadium concentration is preferred (Schaller and Triebig, 1987; Alessio et al., 1988).

Vanadium in the environment

Reviews have been made by World Health Organization (1988) and Byerrum (1991).

Some vanadium has been found in the air over even relatively unpopulated areas of the earth. Sources of vanadium in ambient air are combustion of coal crude oils, and undesulphurized oils. Twice as much vanadium appears in the air during the heating season, and regional variations occur. Annual averages of 60 ng/m^3 seem typical for large American cities (Chicago); there is a marked seasonal variation, the mean for winter months (first quarter) being 6 times higher than for summer months (third quarter) (120 ng/m^3 versus 20 ng/m^3). A detailed analysis of the existing information on vanadium concentrations in air has been made by EPA (1977). It has been estimated that about 1 μg of vanadium could enter the respiratory tract per day if air concentrations are assumed to be about 50 ng/m^3.

The concentrations of vanadium in water depend largely on geographical location and may range from about 0.2 to more than 100 μg/L. In drinking water supplies in the United States, 91% of samples analyzed had below 10 μg/L, and the average was 4.3 μg/L. However, typical values in drinking water appear to be about 1 μg/L.

Vanadium concentrations in different soils as reported by several authors vary between about 5 and 140 mg/kg, and may reach very high values when the soils are polluted by fly-ash (up to 400 mg/kg) (Lagerkvist et al., 1986).

Vanadium is ubiquitous, so it is not surprising that it is contained in the foods that humans consume. Older data are reported in the mg-range (Schroeder et al., 1963); recent data show that it is likely that vanadium concentrations in common food do not exceed a few μg/kg (NAS, 1974). Data on meat are of the order of 1 μg/kg (Byrne and Kosta, 1978). Nevertheless, food is the main source of vanadium intake for man. The daily human intake of vanadium from food is in the order of few tens of micrograms (Byrne and Kosta, 1978). In other studies daily intakes of 8.2 – 13.2 μg (mean 10.7 μg) and 12 – 28 μg have been reported (Schaller and Triebig, 1987; Erdman et al., 1984).

Metabolism and toxicity of vanadium and its compounds

Metabolism

Vanadium is mainly absorbed via the respiratory and gastrointestinal routes. The oxidation state of vanadium does not appear to influence absorption (Carson et al., 1986). In occupational exposure the main route of absorption is by inhalation of dust and fumes containing vanadium or vanadium compounds.

The extent of absorption of different vanadium compounds in the lungs has not been adequately determined although it is estimated that about 25% of soluble vanadium compounds are absorbed (ICRP, 1975). Vanadium pentoxide (V$_2$O$_5$) as well as

vanadiumoxychloride (VO_2Cl) are absorbed nearly totally via the lungs (Conklin et al., 1982).

NAS (1974) stated that environmental exposure for vanadium via the dermal route is apparently of minor importance. Cutaneous absorption seems to be of no significance in man during exposure at the workplace.

Gastrointestinal absorption is the major route for nonoccupational exposure. The major source of gastrointestinal intake is diet. Absorption of the quantity introduced with the diet is usually low (< 1 percent) (Byrne and Kosta, 1978). Experiments in which rats were given radio-vanadium compounds by mouth showed intestinal absorption of only 0.5% of the amount administered.

Ingested vanadium is mainly eliminated in the faeces while the kidneys are the principal route for elimination of that vanadium which is absorbed (Talvitie et al., 1954; Roshchin, 1988). The amount eliminated via the urine was estimated to be about 40 to 60% within 1 to 3 days after resorption. A minor portion of about 10% is excreted via the faeces (Conklin et al., 1982; Talvitie et al., 1954; Roskin, 1968). Absorbed vanadium is transported mainly in the plasma and is widely distributed in body tissues. In unexposed humans, 95% of the blood vanadium is in the transferrin of the plasma. Short-term localization in liver, spleen kidney and lung was reported by Roskin (1968) in animal feeding experiments. In animal experiments vanadium was accumulated especially in bone, but also in liver, lung, kidney and placenta (Conklin et al., 1982; Talvitie et al., 1954). According to Parker et al. (1980) and Sharma et al. (1980) liver, kidneys, bone, spleen and testes are the organs which display the longest half-times.

Toxicity

Vanadium can cause acute as well as chronic effects in human. It is noted that toxicity depends on the oxidation state and augments with increasing states, the most toxic being vanadium (V) such as V_2O_5 or VO_2Cl.

Acute exposure to vanadium compounds causes irritation of the eyes and the upper and lower respiratory tract. Mild effects are characterized by sneezing rhinitis, cough, chest pain and conjunctivitis. These effects seem to be concentration-dependent and usually reversible. Exposure of healthy volunteers to vanadium pentoxide dust (1 mg/m^3) produced respiratory irritation and a persistent cough lasting 8 days; this occurred after about 12 h. Exposure to a lower concentration (0.2 mg/m^3) had the same effect but the induction time for the persistent cough was longer (about 20 h). Concentrations of 0.1 mg/m^3 caused no irritation (Zenz and Berg, 1967).

In severe cases, which are mainly also reversible, bronchitis, obstructive bronchitis and bronchopneumonia may result. Reversible reduction of forced vital capacity and forced expiratory volume was found in boiler cleaners exposed to vanadium-containing respirable dust (Lees, 1980).

A green-black discoloured tongue can be regarded as an indicator of vanadium exposure, but may be absent even with long term exposure.

Chronic exposure to vanadium may also cause rhinitis, pharyngitis and bronchitis, but the amount of exposure is not conclusive. Evidence of systematic effects in workers exposed to vanadium compounds is sketchy. In most cases, no systematic effects have been reported (Symansky, 1939; Williams, 1952; Lewis, 1959; Zenz et al., 1962; Eisler et al., 1968). Other reports describe only vague or unspecific signs and symptoms such as weakness, ringing in the ears, nausea, vomiting, and headache (Roshchin, 1962).

Metabolic effects include interference with the biosynthesis of cystine and cholesterol, depression and stimulation of phospholipid synthesis and, at higher concentrations, inhibitions of serotonin oxidation. A 1981 study did not reveal any decrease in serum cholesterol or increase in serum triglycerides (Kiviluoto et al., 1981).

Reviews are reported by Carson et al. (1986), Lagerkvist et al. (1986), WHO (1988), and Byerrum (1991).

Biological levels without occupational exposure

The principal source of vanadium for the general population is from the low levels found in food. With poor absorption via the gut, vanadium is usually at very low concentrations in the urine (Byrne and Kosta, 1979).

Table 1 shows the urinary levels of vanadium in occupationally non-exposed subjects. It is generally agreed that the normal concentrations are less than 1 μg/L urine.

Table 2 presents the vanadium levels in blood or serum/plasma by various investigators in occupationally non-exposed subjects. The data, especially those obtained by neutron activation analysis, show very low levels.

Estimates of vanadium concentration in the human lung are 0.01 – 1 mg/kg and 0.019 – 0.190 mg/kg. Other soft tissues seem to contain much less vanadium. For

TABLE 1

VANADIUM CONCENTRATIONS IN URINE SAMPLES OF NON-OCCUPATIONALLY-EXPOSED SUBJECTS

Reference	Mean μg/L	Range μg/L	Method
Byrne and Kosta, 1978	0.30		NAA
Ishizaki and Ueno, 1979	0.26		AAS
Buchet et al., 1982	0.44	0.3-0.7	AAS
Cornelis and Versieck, 1982		0.033-0.113	NAA
Maroni et al., 1983	0.8 ±0.72		NAA
Buratti et al., 1985	0.36±0.15		AAS
Apostoli et al., 1988	0.29±0.17		AAS
Minoia et al., 1990	0.8 ±0.08	0.2-1.0	AAS
Fleischer et al., 1991	0.33	0.1-0.68	Voltammetry

TABLE 2

VANADIUM CONCENTRATIONS IN BLOOD/SERUM SAMPLES OF NON-OCCUPATION-ALLY-EXPOSED SUBJECTS

References	Mean μg/L	Range μg/L	Material	Methode
Byrne and Kosta., 1978	0.5		blood	NAA
Glyset et al., 1979	0.78		blood	NAA
Cornelis et al., 1980	0.033	0.024-0.939	serum	NAA
Godin, 1990	1.32	0.58-3.95	serum	HPLC
Maroni et al., 1983	0.94±0.65		blood	NAA
Simonoff et al., 1984	0.670	0.26-1.3	blood	NAA
Minoia et al., 1990	0.62±0.03 0.35±0.11 (N=65)	0.07-1.1 0.09-0.75	serum	AAS

example, the brain, muscle and liver concentrations have been reported to be about 7, 0.5 and 6 μg/kg (Byrne and Kosta, 1978).

Vanadium concentrations in the lung and in hilus tissue of non-exposed persons ranged from 9 to 40 μg/kg wet weight. Vanadium was inhomogeneously spread over the lung. The highest vanadium concentrations have been found in the upper lung areas.

ANALYTICAL INTRODUCTION

For monitoring occupational and environmental exposure to vanadium and its compounds, measurements of vanadium in body fluids, excreta and tissues from human beings are carried out (Alessio et al., 1988; Schaller and Triebig, 1987; Kraus et al., 1989). The biological indicator levels are influenced by the chemical and physical properties of the vanadium compound studied and by the time of sampling. Since the analyst is faced with detecting vanadium in the lower μg/kg range, contamination control and choice of analytical procedure is of fundamental importance for an adequate monitoring program.

Types of specimens and preanalytical phase

Vanadium concentrations in blood, serum or urine are used as a biological indicator of exposure to vanadium. Urine and serum are the specimens with widest application and greatest practicability for monitoring human exposure to vanadium compounds, but urine is preferred as an indicator medium. Blood vanadium appears to be a less sensitive indicator than urinary vanadium, partly because the differences in concentrations are hardly appreciable at low levels of exposure with the analytical methods available (Alessio et al., 1988).

The vanadium contents in tissue specimens (mainly in the lungs) is important only when it has to be decided whether an occupational disease has been caused by an occupational exposure to vanadium and/or its compounds (Kraus et al., 1989).

For vanadium determinations, there is a growing recognition of the importance of the preanalytical phase (specimen collection, transport and storage) for unbiased results. The overall abundance of vanadium in the human environment requires a strict contamination control in the preanalytical phase as well as in the analytical phase. This is important as vanadium is among the least concentrated elements in biological materials. Collection of urine and blood specimens should take place in a non-contaminated area after showering and changing into street clothing. Urine (around 50 mL) should be voided directly into acid-washed plastic containers. Standard collection equipments, e.g. Vacutainer[R] and Monovetten[R] (EDTA-K as anticoagulant) can be used after checking all sources for possible contamination for blood sampling. For most analyses 5 mL whole blood is sufficient. From blood samples serum/plasma is often separated and its vanadium content is determined, because vanadium is mainly in blood serum/plasma (Gylseth et al., 1979; Cornelis et al., 1980).

Tissue specimens should be homogenized before digestion. Sunderman et al. (1986) have shown that this is possible without contamination using a "stomacher" blender. The pretreatment of biological tissues usually involves digestion of the biological matrix. Ashing in furnaces is not very suitable for biological specimens because of sputtering, volatilization and contamination. Wet oxidative digestion in open and closed quartz or PTFE vessels are preferred. Because of the omnipresence of vanadium it is irrevocably necessary to analyze blank values within each series of analyses.

Analytical techniques

Numerous methods have been described for the determination of vanadium in biological materials. Spectrophotometric methods were formerly used for analysis of vanadium, e.g. the 8- hydroxyquinoline method (for reviews see NAS, 1974; Berman, 1980). Fishman and Skougstad (1964) proposed a procedure to determine vanadium in water by means of a method which is based on the oxidation reaction of gallic acid catalyzed by vanadium. The method has been applied to biological materials (Welch and Allaway, 1972), blood (Kelm and Schaller, 1978) and urine (Holzhauser and Schaller, 1977; Thürauf et al., 1979). But spectrophotometric methods generally require adequate pretreatment of samples (such as acid digestion), masking or removal of interfering cations to assure a definite valency state of vanadium, and often an extracting agent. In all these pretreatment steps contamination may occur. Furthermore the sensitivity of these methods does not allow the determination of vanadium levels in the normal range.

Physical methods utilizing neutron activation, atomic emission spectrometry, graphite furnace atomic absorption spectrometry and adsorptive inverse voltammetry are presently used. Neutron activation determination seems to be the most reliable method for the analytical determination of vanadium in biological specimens taken from occupationally nonexposed and exposed people (Allen and Steinnes, 1978; Glyseth et al., 1979). In

biological samples, destructive techniques using post-irradiation radiochemical separation are capable of achieving an absolute detection limit of about 0.2 ng vanadium (Byrne and Kosta, 1978). Cornelis et al. (1980) and Simonoff et al. (1984) also used neutron activation analysis for the determination of vanadium in blood. They concluded that this technique appears to be the most sensitive and least biased available for the blood vanadium analysis. However, neutron activation analysis is very cumbersome and restricted to a few laboratories and can not be performed routinely.

At present, the most commonly used techniques for the determination of vanadium are graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma emission spectrometry (ICP-AES) and adsorptive inverse voltammetry (Fleischer et al., 1991).

In the literature there are several reports on AAS methods for the determination of vanadium in biological samples (Krishnan et al., 1976; Stroop et al., 1982; Buchet et al., 1982; Pyy et al., 1984; Buratti et al., 1985; Apostoli et al., 1988; Fleischer et al., 1991; Arbouine and Smith, 1991).

Those methods are based on the GFAAS technique, because the conventional FAAS is not sensitive enough for the determination of vanadium in biological samples.

Urine samples can be determined directly or after preconcentration by mineralization and vanadium chelation followed by solvent extraction.

Pyy et al. (1984) analyzed urinary vanadium after sample dilution and the standard addition method with pyrolytic-coated graphite tubes. The practical detection limit was 2 μg/L. With regards to the vanadium content of serum, amounts of 2.2 – 3.9 μg/L have been obtained by analyzing diluted samples directly (Stroop et al., 1982). Fleischer et al. (1991) proposed a GFAAS method using matrix modification for vanadium determination in urine. The matrix modifier is made up by sodium fluoride, tetrammine-palladium(II)-nitrate and magnesium nitrate. The detection limit is around 1 μg/L, the precision 2% and the recovery rate 97.5%. A highly significant correlation was evaluated for the comparison with a ICP-OES method and a voltammetric method.

To improve the detection limits and to avoid matrix interferences the preparation of biological samples usually involves vanadium chelation followed by solvent extraction.

Buchet et al. (1982) combined wet ashing of the urine with nitric acid with cupferron-chelation and 4-methylpentan-2-one extraction. Cupferron gave the best results in comparison to ammonium pyrrolidine dithiocarbamate (APDC) and 5,7-dichloro-8- quinoli-nol (dichloroxine).

Apostoli et al. (1988) described a GFAAS method combining solvent extraction (cupferron-MIBK system) with preheating the graphite tubes (at 150°C) before sample injection. A tenfold increase in sensitivity was achieved together with an improvement in analytical accuracy. The detection limit is 0.5 – 1 μg/L with injection at room temperature (20 μL sample), 0.1 – 0.5 μg/L with injection at 150°C (20 μL sample) an 0.05 – 0.1 μg/L for the 150°C injection using a 40 μL sample.

A GFAAS method for determining levels of urinary vanadium as low as 0.1 μg/L is described by Arbouine and Smith (1991). Vanadium is chelated with cupferron and ex-

tracted in MIBK. Residual matrix interferences are overcome by using matrix matched standards.

Other authors propose the APDC/MIBK chelation/extraction system for the vanadium determination in urine by GFAAS (Buratti et al., 1985; White et al., 1987). A detection limit of 0.4 μg/L is reported.

Atomic absorption is also widely used for the determination of vanadium in tissues and serum. Digestion procedures prior to graphite furnace procedures are necessary. A detection limit of 30 pg and a sensitivity of 65 pg have been reported (Stroop et al., 1982).

Plasma atomic emission spectrometry, e.g. DCP-OES, represents a technique of instrumental methods for the analytical determination of vanadium.

DCP-OES procedure for animal tissues have been published in which the lower limit of the working range for vanadium is 3 μg/L (Frank and Petersson, 1983). Urine could be analyzed directly for vanadium levels of 10 μg/L, where the method of standard addition was used. For the determination of low vanadium concentrations in urine and serum, the use of an extraction method with APDC into MIBK was necessary. The detection limit was about 2.5 μg/L (Pyy et al., 1983).

ICP-OES is the most practical technique for vanadium determination, with a detection limit of around 5 μg/L urine. The precision is 8.5% and the recovery 98%. The urine is acidified with nitric acid (4.5 mL urine and 0.5 mL HNO_3, 65%) and without further sample processing directly transferred into the nebulizer of the spectrometer. Aqueous standards are used for calibration (Schramel, 1993).

The adsorptive inverse voltammetric determination of vanadium is a very sensitive method with detection limits of 0.2 μg/L. After a complete wet oxidative digestion of the sample the VO_{2+} or VO_{3+} is complexed with cupferron by pH 8.5. The complex is adsorbed over a defined period at a hanging mercury drop (HMDE). Subsequently a potential range of −0.5 V to −0.95 V was applied in the differential pulse mode to measure the peak current at around −0.75 V. The quantitative determination was carried out by means of the method of standard addition using at least three additions. The precision in series is 6.4% at a concentration of 2.5 μg/L urine and the day to day precision 3.9% at a concentration of 5 μg/L urine (Seiler, 1993). A detailed description of this sensitive method is given in the 4th volume of "Analyses of Hazardous Substances in Biological Materials" (Angerer and Schaller, 1993).

ANALYTICAL PROCEDURE

Determination of vanadium in urine

For risk assessment in the case of vanadium uptake urine is the matrix of choice. The collection of urine is non-invasive and is practical under routine conditions. Moreover this parameter is more sensitive for diagnostic purposes than the vanadium concentration in blood. A tentative biological threshold limit value of 50 mg/kg creatinine has been proposed for urinary vanadium (Lauwerys, 1983).

GFAAS with chemical matrix modification enables a sensitive vanadium detection with acceptable expenditure of time and work. The following detailed working procedure is recommended (Fleischer et al., 1991; Angerer and Schaller, 1993).

Principle of the method

Vanadium is determined in the urine without digestion by Zeeman GF-AAS according to the standard addition procedure. Pyrolytically coated graphite tubes are used. In order to reach optimum conditions of atomisation and mineralisation a mixture of tetrammine-palladium(II)-nitrate/magnesium nitrate/sodium fluoride is added to the sample.

The addition of the Pd/Mg modifier causes a sharp rise and increase of the signal. By adding sodium fluoride the form of the atomisation peak is optimised.

The addition of the modifier mixture leads to optimised conditions of atomisation whereby the detection limit can be increased further by the evaluation at peak height.

Instrumentation and reagents

Atomic absorption spectrometer with Zeeman correction
Graphite furnace with autosampler and pyrolytically coated graphite tubes
Monoelement vanadium hollow cathode lampe

Vanadium standard, 0.1 g/l (NH_4VO_3 in alkaline solution)
Triton[R] X-100
Tetrammine-palladium(II)-nitrate
Magnesium nitrate
Sodium fluoride

Solutions:
Calibration standards: 150 μg/L, 300 μg/L, 450 μg/L
Modifier I: Sodium fluoride, 2% aqueous
Modifier II: Tetrammine-palladium(II)-nitrate, 2%; magnesium nitrate, 0.5% and Triton[R] X-100, 0.1% in one solution
Buffer: 0.02 M NH_3/NH_4Cl

As in all trace element analyses, reagents must be of highest purity, and glassware and tubes scrupulously clean.

Analytical procedure

The urine is collected in polyethene bottles, and the specimens are refrigerated (up to 7 days) or deep frozen until use. Specimen collection must be carried out as described in Sec. Sampling and Sampling Storage (A. Aitio and J. Järvisalo). Contamination must be strictly avoided. The sample preparation for the AAS analysis including standard addition

TABLE 3

SCHEME FOR THE SAMPLE PREPARATION AND STANDARD ADDITION METHOD

Sample (mL)	Modifier I (mL)	Modifier II (mL)	Buffer (mL)	Water (mL)	Standard I (mL)	Standard II (mL)	Standard III (mL)
0.5	0.1	0.1	0.05	0.25			
0.5	0.1	0.1	0.05	0.15	0.1		
0.5	0.1	0.1	0.05	0.15		0.1	
0.5	0.1	0.1	0.05	0.25			0.1
Blank	0.1	0.1	0.05	0.75			

Standard I: 150 μgV/L; II: 300 μgV/L; III: 450 μgV/L

TABLE 4

OPERATIONAL PARAMETERS FOR GFAAS (instrument dependent)

Atomic absorption spectrometer:
Wavelength: 318.4 nm
Background correction: Zeeman compensation
Spectral slit width: 0.7 nm
Analytical determination: peak height
Inert gas: Argon
Injected volume: 20 μL
Temperature-time programme

Step	1	2	3	4	5	6	7	8
Temperature	110	350	1500	1500	2700	20	2700	20
Ramp Time	10	10	10	1	0	1	0	1
Hold Time	20	5	20	9	5	9	5	5
Record					X	X	X	
Read					X			
Intern Flow					o			

technique should be carried out as summarized in Table 3. Table 4 shows the operational parameter for the AAS conditions. The data may serve as a guide. However, an optimisation of the temperature-time programme must be carried out for each individual instrument.

Reliability of the method

The within-series imprecision of the method is 2% for an average vanadium concentration of 10.2 μg/L. The mean recovery rate was calculated to 97.5% and the analytical detection limit calculated as three times the standard deviation of the blank around 1 μg/L. Parallel determinations of the urine samples with an adsorptive inverse voltammetric method (Seiler, 1993) resulted in a coefficient of correlation of 0.992 (y' = 1.03 x − 0.17), the comparison with the ICP-OES method (Schramel, 1994) a correlation coefficient of 0.986 (y' = 0.96 x + 1.97) in a concentration range from 1 to 25 μg/L (Fleischer et al., 1991).

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Chapter 25

Zinc

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INTRODUCTION

The fundamental importance of zinc in most aspects of intermediary metabolism has been uncovered with the help of a variety of powerful analytical techniques. Over a hundred zinc metallo-enzymes have been isolated in plants, animals and micro-organisms. The structure of some of these metallo-proteins has been described using X-ray crystallography, nuclear magnetic resonance and electron probe microanalysis. These zinc-containing enzymes are needed in almost all stages of both nucleic acid and protein synthesis. Vallee and Falchuk (1981) have studied the effect of zinc deprivation on the growth of euglena gracilis and consider that zinc-histone complexes regulate gene expression. There is recent interest in the role of "zinc fingers" in DNA binding domains (Harrison, 1991). Clearly zinc is an integral part of the most vital of cellular processes. Lack of this essential trace metal causes a reduction in cell division and resultant failure of growth, weight loss and impairment of tissue repair in the whole animal. The practical importance of zinc nutrition is understood in agriculture; for an adequate supply of the metal is needed for the efficient production of both plant and animal crops (Underwood, 1977). The role of zinc in human nutrition is less well accepted in spite of increasing evidence as to its importance (Prasad, 1988).

ZINC METABOLISM - AN OUTLINE

As with other essential nutrients there are homeostatic mechanisms which maintain a constant tissue concentration of zinc in spite of fluctuations in dietary supply. The total dietary intake is 10-15 mg per day. The bioavailability of zinc from different foodstuffs varies. Some 40% of zinc is absorbed from the average diet. Inside the intestinal mucosal cell zinc enters a metabolic pool in equilibrium with zinc-thionein. The synthesis of this metal binding protein is induced by various metals, and it appears to regulate their intracellular transport. Zinc leaves the intestinal mucosal cell across the plasma membrane and is taken up by albumin in the portal circulation. The liver extracts zinc with a high

efficiency and within the hepatocyte the metal is utilised for the synthesis of the numerous zinc metallo-enzymes. Zinc is exported from the liver to all metabolically active peripheral tissue. The transport form in peripheral blood is probably a zinc albumin complex (80% of total plasma zinc) in equilibrium with amino acid zinc complexes (< 2% of total plasma zinc). Plasma zinc uptake by tissue cells is not understood in detail, but it is thought there is a small mobile intracellular pool able to exchange with the plasma albumin zinc complex.

Zinc is excreted from the body primarily in the faeces. There is a substantial enterohepatic re-circulation, and as Cousins (1982) notes, the gut gets two zinc meals per day, one from foodstuff, the other from zinc re-entering the intestinal lumen in digestive juices and crossing the mucosal barrier from the plasma zinc-albumin complex. The total zinc output in faeces usually equals the dietary intake at around 10-15 mg per day.

Urinary excretion of zinc is much smaller at 0.5 mg zinc per day. Urinary output does not increase as dietary zinc intake rises.

DISORDERS OF ZINC METABOLISM

It is important to distinguish between changes in zinc metabolism which occur as a secondary effect of disease, injury, infection and drug therapy and alterations caused by a primary nutritional zinc deficiency. There is confusion in the literature because a number of unrelated causes can temporarily lower the concentration of zinc in plasma, and this is reported uncritically as evidence of nutritional depletion. Since a high proportion of zinc in plasma is albumin bound, any circumstance which lowers plasma albumin will also lower plasma zinc. For example, the changes seen in severe liver disease are primarily caused by a failure of hepatic synthesis of plasma proteins such as albumin. This results in problems in the distribution of zinc and eventual tissue depletion. It is questionable whether zinc supplementation of diet is worthwhile without some restoration of hepatocyte function, by effective treatment of the underlying disease (Mills et al., 1983).

Serious disease of the intestinal tract may disturb zinc absorption and increase faecal zinc excretion. Inflammatory bowel disease with persistent diarrhoea as in Crohn's Disease could have complicating features due to a secondary zinc deficiency (Sandstead, 1982).

There is a large increase in urine zinc whenever there is accelerated breakdown of skeletal muscle and other tissue. After injury or in acute starvation the rise in urinary zinc is evidence of a catabolic state (Fell et al., 1973).

As stated earlier plasma zinc concentration is low in a wide range of conditions (Halstead and Smith, 1970). One effect of 'stress', mediated by ACTH and cortisol, is to reduce plasma zinc. This is part of the acute phase response to accidental or surgical injury, the effect lasting for several days. Bacterial or viral infections have a similar effect. There is recent evidence that the fall in plasma zinc is part of a complex series of metabolic events induced by the humoral factors such as Interleukin 1 (Dinarello, 1982) which in turn activates interleukin-6 the active factor responsible for induction of liver

metallothionein and subsequent removal of zinc from the plasma albumin fraction (Schroeder and Cousins, 1990).

BIOCHEMICAL AND CLINICAL PRESENTATION OF ZINC DEFICIENCY

Lack of zinc has a variable effect upon the activities of the zinc containing tissue enzymes. Golden and Golden (1981a) note that overall the biochemical changes resemble those seen in essential amino acid deficiency (Table 1).

The resultant pathology and eventual clinical presentation of signs and symptoms must have their origins in these biochemical lesions. However, the detailed mechanisms underlying the clinical effects of various degrees of zinc deficiency are not understood. The presenting features are non-specific (Table 2). Rapidly dividing tissue cells such as those in the intestinal mucosa and in skin epithelium are sensitive to zinc depletion.

TABLE 1

SELECTED BIOCHEMICAL CHANGES AND OTHER LABORATORY INDICES IN EX-PERIMENTAL ZINC DEFICIENCY. Abstracted from Prasad (1982)

Zinc dependent enzyme systems

Alkaline phosphatase activity reduced in plasma - bone - intestine - kidney - stomach - red cell membranes - neutrophils
Carbonic anhydrase activity - reduced in intestine - red cells
Alcohol dehydrogenase activity - reduced in liver - bone - testes- kidney - oesophagus
Deoxythymidine kinase activity - reduced in connective tissue collagen

Protein metabolism

Protein synthesis reduced, protein catabolism increased
Total protein content of tissue reduced
Polysome patterns abnormal
S^{35} incorporation into protein reduced
Plasma protein synthesis reduced - low concentration of plasma albumin-prealbumin-retinol binding protein
Plasma ammonia concentration increased

Nucleic acid metabolism

Biosynthesis and catabolism of DNA and RNA altered
Thymidine incorporation into DNA reduced
DNA polymerase activity reduced - E.coli
P^{32} incorporation into RNA reduced

TABLE 2

CLINICAL PRESENTATION OF ZINC DEFICIENCY

Loss of appetite, loss of sense of taste and smell
Restriction of growth - dwarfism
Failure of sexual maturation - hypogonadism - testicular function impaired
Skin lesions - acrodermatitis
Delayed wound healing
Hair loss - alopecia
Diarrhoea - abdominal colic
Behavioural changes - depression
Recurrent infections - suppression of immune response

OCCURRENCE OF HUMAN DEFICIENCY

Primary zinc deficiency severe enough to cause an obvious clinical disorder is rare. Indeed, the widespread occurrence of zinc in most foodstuffs was thought to make such a condition improbable. However, Prasad and Halsted (1961) identified a disorder in adolescent males in Middle Eastern populations, presenting as a complex syndrome of hypogonadal dwarfism, as a nutritional disease which responded more rapidly when extra zinc was given in the rehabilitation diet. The problem was the low bioavailability of zinc from the cereal rich diet. It is now considered that up to 3% of adolescents in Middle Eastern countries have some degree of growth retardation due to zinc depletion and that pregnant and lactating women are also at risk in these areas (Casey and Hambidge, 1980). Studies by Golden and Golden (1981b) of cases of marasmus and kwashiorkor in the West Indies show that the amount of available zinc given in the rehabilitation diet not only was the limiting factor for growth but also determined the proportion of adipose or lean body tissue laid down by the recovering children.

A rare inborn error of metabolism, acrodermatitis enteropathica, is now known to cause intestinal malabsorption of zinc (Moynahan, 1974). This previously fatal disorder presents in young children after weaning from breast milk. Clinical signs include an intractable rash around the oral, nasal and anal regions, as well as the genitalia. The child loses weight, has severe diarrhoea and many of the other features listed in Table 2. Treatment with oral administration of zinc sulphate, in sufficient dosage to overcome the intestinal block, has a remarkable effect, the patient responding within a few weeks of treatment.

A very similar clinical presentation has been noted in adults treated for relatively prolonged periods by total intravenous nutrition, using nutrient mixtures with insufficient zinc (Kay et al., 1976). It is now accepted that zinc should be added to the nutrient mix from the start of treatment. We have found that although patient requirements can vary considerably, addition of 6.5 mg per day is effective and safe.

As discussed above, a wide range of much more common conditions may so disturb the regulation of zinc metabolism that a degree of secondary zinc depletion results. In these cases, the effects will be difficult to separate from those caused by the primary disease, but there is interest in the role of zinc in relation to cellular immunity (Good, 1981). Disorders of the special senses such as taste and smell are also linked to marginal zinc depletion (Russell et al., 1983).

LABORATORY INVESTIGATION OF ZINC METABOLISM

Since the presentation of zinc deficiency is so variable and non-specific, laboratory measurements are required, along with clinical observation, to substantiate a diagnosis.

The direct determination of zinc in diet, tissue and in body fluids can be accomplished by a variety of methods. A common limitation is the chance of sample contamination prior to analysis. Some early studies using less sensitive methods may not have recognised this problem and reported erroneously high results. Older colorimetric methods required that the biological sample be efficiently digested or otherwise deproteinised, prior to formation of a coloured zinc complex. These techniques have largely been superseded by atomic absorption spectrometry which is more sensitive yet less prone to interferences. For fluids such as plasma or urine, simple dilution is all that is required prior to analysis. Tissue or diet samples only require to be dissolved in mineral acid. These simpler sample preparation procedures limit the chances of contamination.

For metabolic studies, zinc isotopes can be used and both stable and radioactive forms are available. Isotopic methods can give valuable insights into the dynamics of zinc absorption, tissue uptake and excretion.

The numerous biochemical pathways which are zinc dependent, can be investigated. Some of the effects listed in Table 1 have been used in the diagnosis of deficiency and can provide complementary evidence although the results can also be affected by other factors.

LIMITATION OF CONTAMINATION

In the summaries of methods which are presented it is assumed that the following general precautions will be taken:
1. The sample collected by a procedure demonstrated by the analyst to cause minimal 'blank signal'.
2. Deionised/distilled water to be used for all purposes and to be checked regularly for possible zinc contamination.
3. All glassware or plastic ware to be acid washed, rinsed in pure water, oven dried, and stored in dust-free conditions.
4. A suitable grade of 'metal free' mineral acid or other reagents used throughout.

DETERMINATION OF DIETARY ZINC

Since foodstuffs include a wide range of biological materials, the method of zinc analysis must be able to deal with physically diverse samples, ranging from liquids and soft foods through to meat or other foods which may be difficult to homogenise. Ideally, a duplicate diet will be collected for a reasonably representative period - at least 5 days. The total diet, collected in a suitable plastic container, is then homogenised, using the 'Colworth Stomacher' type of apparatus, which does not require the diet to come in contact with metal homogeniser blades. Since the zinc content of different foods varies widely and also varies between different samples of the same class of food (Table 3), it is preferable to attempt direct analysis rather than derive the daily intake from food tables.

As most foods contain at least 5 mg/kg dry weight, a sample of approximately 1 g of dry food dissolved in acid and diluted to 25 ml in water, will contain about 0.2 mg Zn/L, which can be easily determined by flame AAS.

TABLE 3

Food	Zn concentration mg/kg dry weight	Zn concentration mg/kg wet weight
Meat	5-202	4-87
Liver	94-257	39-78
Kidney	94-139	19-78
White fish	18-23	3-6
Green vegetables	7-54	0.1-0.6
Potatoes	10-11	0.2-0.6
Wholemeal flour	35	30
White flour	8	7
Cow's milk	16-48	2-6

(Abstracted from McCance and Widdowson, 1976)

Dry Ashing Procedure

A portion of the homogenised diet is taken and the wet weight and dry weight of approximately 1 g (dry) is determined. The dry material is placed in a crucible with a fitted lid. Platinum crucibles, if available, are excellent. Silica crucibles or borosilicate beakers are satisfactory but should be discarded if they become etched. Porcelain crucibles are not recommended.

The organic matter is destroyed by ashing overnight in a furnace at 450°C, and zinc is not volatilised at this temperature. The ash residue is taken up in 2 mL of 6M hydrochloric acid and then this is evaporated to dryness, this process is then repeated to ensure the

hydrolysis of any pyrophosphates present. The final residue is treated with 0.5 mL 6M hydrochloric acid, diluted with water, warmed, and made up to a final volume of 25 mL. Samples which resist this digestion procedure can be re-ashed overnight, perhaps using an ashing aid such as concentrated nitric acid. Any small insoluble residues, such as silica from plant material, can be removed by filtration.

Wet Ashing Procedure

A portion of diet homogenate, equivalent to 1 g dry weight, is placed in a Teflon beaker and an accurate weight taken. Nitric acid (15 mL, conc. Aristar) is added and the mixture digested at low heat. After the initial frothing has subsided, the temperature is increased to reduce the volume to about 5 mL, at which point perchloric acid (5 mL conc. Aristar) is added. The mixture is then heated strongly until the appearance of white fumes of perchloric acid and the volume is reduced nearly to dryness. If there is substantial charring and the solution turns black, further amounts of nitric acid are added and ashing repeated until a clear solution is obtained. The acid digest (around 0.5 mL) is diluted to 25 mL with water.

If the diet or foodstuff has a particularly high fat content, then sulphuric acid is added to the digestion mixture. This is a potentially dangerous procedure and where possible dry ashing should be used for such samples.

Flame AAS Determination

The diluted acid digest is aspirated into an air-acetylene flame and the zinc absorbance at 213.9 nm recorded. Calibration standards in the range 0.1-1.0 mg/L are prepared from stock BDH solution of 1 g/L in 0.1 M hydrochloric acid for the dry ashed samples or in 0.1 M perchloric acid for the wet ashed samples. It is necessary to use a background correction system since there can be a variable amount of non-specific scatter.

BIOAVAILABILITY OF ZINC FROM DIET AND THE RECOMMENDED DIETARY AL-LOWANCE

The results of dietary zinc analysis need to be considered in terms of the availability of the zinc in the food for intestinal absorption. The zinc content of whole meals and the total daily zinc intake are not sufficient information on their own, without knowledge of factors which inhibit or promote intestinal absorption (O'Dell, 1984). Free ionic zinc probably does not exist in the intestinal tract, zinc being bound to molecular species such as protein, amino acids, phytic acid, citrate and others. The bioavailability of the metal is determined by the nature of these zinc binding ligands. When the zinc complex is insoluble as in Zn-phytate, the uptake from diet is poor, whereas zinc-protein or zinc-amino acid complexes are more easily dissociated and are a good source of available zinc. Other dietary components affect zinc absorption such as the amount of iron, calcium and phosphate.

When the ratio of the concentration of these substances to zinc is excessive, zinc is poorly absorbed.

Assessment of Zinc Bioavailability

A number of methods have been used:

1. Absorption experiments in which the fraction of a known amount of a zinc tracer, usually Zn^{65}, crossing the intestinal wall is measured. The tracer can be added as an inorganic salt and mixed into the diet very thoroughly; this is *extrinsic* labelling. Or the label can be incorporated into the growing plant or animal crop and thus into the processed foodstuff; this is *intrinsic* labelling.
   A volunteer consumes the labelled food as part of the diet under study and the whole body radioactivity is determined in an appropriate whole body counter, and this is repeated after 14 days, by which time all non-absorbed material will have been eliminated in the faeces. The fraction of the original activity retained gives a measure of zinc absorption from that diet.

2. Zinc metabolic studies in which the difference between the total dietary intake and the total excretion in faeces and in urine, is measured over a period of several days. The difficulties of this procedure include problems of ensuring complete collections of excreta and measurement of losses in sweat, shed skin and hair. These losses al-though minor, may cause cumulative errors. Analytical problems caused by accidental zinc contamination are also a worry, although here the use of stable zinc isotopes and mass spectrometry or neutron activation is an advantage.

3. A biological measure of the effect of different diet on the growth and well-being of experimental animals.

4. The direct measurement in the diet of substances known to adversely affect zinc absorption. This most commonly would require determination of the phytic acid cont-ent. Substances such as dietary fibre, calcium, iron, tin, oxalate and others, are also important in certain instances.

Determination of Dietary Phytate

The methods for phytate assay have been reviewed by Oberleas (1971). The usual procedure is an iron precipitation, in which the phytate extracted from food by acid is precipitated by an excess of Fe(II). The iron in the supernatant or in the precipitate is measured and the phytate content determined by comparison with pure phytate standards. A highly specific NMR method has recently been used (O'Neill et al., 1980).

Phytate to Zinc Ratio

Cereals, pulses and soya bean products are particularly rich in phytate. The [phytate]:[zinc] molar ratio has been used as an index of the bioavailability of zinc from different foods. Diets where the ratio is 20:1 or more have been suggested as a cause of human

zinc deficiency (Oberleas and Prasad, 1976). A selection of some cereal based foods are listed in Table 4, and their [phytate]:[zinc] ratio is shown.

It must not be concluded that all cereal products with a ratio of 15-40 are by necessity very poor sources of available zinc. Sandstrom et al (1980) have shown that the total amount of zinc retained from a diet of whole meal bread (ratio 23:1) was greater than that retained from a meal of white bread (ratio 3:1).

The absorption of zinc from any given diet is related to the total amount of zinc present, as well as the amounts of antagonists like phytate and iron, and also to the amounts of protein or other substances which enhance zinc absorption.

TABLE 4

Cereal	Zn concentration mg/kg dry weight	% Phytate	Phytate:Zinc molar ratio
All Bran	80	2.59	32.5
Bran flakes	26	1.1	43.6
Muesli	24	0.86	35.7
Wholemeal bread	29	0.62	21.6
Special K	16	0.15	8.2
Rice krispies	8.3	0.11	12.8
White bread	9.5	0.06	5.9
Corn flakes	2.6	0.03	12.3

Recommended Dietary Intake of Zinc

The dietary allowances proposed in the USA are, for infants 0-1 years, 3-5 mg/day; children 1-10 years 10 mg/day; males and females 11-51 + 15 mg/day; pregnant women 20 mg/day and lactating women 25 mg/day. (Recommended Dietary Allowances 1980). Some countries have adopted these allowances while others like Canada, Australia, GDR and the USSR suggest lower amounts in the range 8-15 mg/day, with addition of up to + 13 mg/day for lactating women. The policy in the UK is to make no particular recommendation since dietary surveys do not suggest that there is a widespread lack of zinc in the average diet.

Certainly, the intake as measured directly by diet sampling tends to show that in Western countries the average diet supplies about 10 mg/day, which is below the proposed USA figure of 15 mg/day for an adult (Lyon et al., 1979).

It is not agreed whether the USA recommendation is too high or if there is some degree of zinc malnutrition even amongst apparently affluent nations.

TISSUE ZINC CONTENT

Various estimates have been made of the whole body content, based initially on direct cadaver analysis and more recently by isotope dilution techniques (Kennedy et al., 1978). Values between 1-2 g for an adult are quoted. Since the greatest amount of zinc is found inside cells, the average tissue concentration is higher than that found in body fluids. Zinc is present in all metabolically active tissue and some results obtained from tissue obtained at autopsy of accident victims, are shown in Table 5. These were obtained using neutron activation analysis (Smith, 1967) and by flame AAS (Lyon et al., 1989, Martin et al., 1992).

It is more convenient to determine soft tissue zinc by AAS by the same procedures described for food samples. Bone and teeth can be ashed similarly but the high concentration of dissolved calcium and phosphate makes it important to use background correction, even with flame AAS. The conventions used to report the analytical results need to be noted. In a compilation of the literature on the elemental composition of human adult tissues, Iyengar et al. (1978) quote values as μg/g fresh weight (i.e. wet weight), dry weight and ash weight. Others seek to relate the trace metal result to another intracellular component such as protein or DNA. Wherever possible, results should be reported in as many of these ways as practicable to allow comparison with other publications.

Determination of Zinc in Biopsy Samples

The needle biopsy makes a variety of tissues available for study. Liver, skeletal muscle, kidney and iliac crest bone can be obtained and have been used in research. Liver biopsy is the most widely used of these procedures since this is a large and fairly homogenous organ, and the technique is widely employed for the histopathological diagnosis of common liver disorders.

Example of the Determination of Zinc in Liver Biopsies (Mills et al., 1983)

The biopsies were taken with a 'Trucut' needle from adjacent sites in the liver, using either a percutaneous method or under direct vision at laparotomy. Preliminary studies using autopsy liver had shown that this type of needle did not cause zinc contamination of the sample.

The biopsy tissue was placed into an airtight plastic envelope and frozen immediately. All samples were stored at -20°C and processed as a single batch. The wet weight was taken (average 25.5 mg), the sample dried at 100°C and the weight recorded, and then the dried material was ashed at 550°C in a muffle furnace.

The residues were dissolved in concentrated nitric acid (0.25 mL, Aristar grade), the volume was made to 10 mL with water and the zinc and magnesium content measured by flame AAS. Liver samples were obtained from patients with alcoholic cirrhosis and from patients undergoing elective surgery who formed a control group.

TABLE 5a

TISSUE ZINC CONTENT OF ACCIDENT VICTIMS (WEST OF SCOTLAND)
Determined as mg/kg dry weight, by neutron activation analysis (Smith, 1967)

Tissue	Number	Range	Mean	Standard deviation
Adrenal	12	25-72	43.2	12.9
Bone	7	15-129	94.0	25.8
Brain	15	20-64	39.1	15.4
Heart	23	59-126	87.9	20.2
Kidney	15	64-320	188	82.6
Liver	21	42-298	169	58.6
Muscle	11	114-246	200	42.9
Prostate	6	126-441	243	—
Skin	3	4-61	25.0	—

TABLE 5b

TISSUE ZINC IN HEALTHY ACCIDENT VICTIMS (WEST OF SCOTLAND)
by flame atomic absorption spectrometry (Lyon et al., 1989) (mg/kg dry wt.)

Tissue	Number	Range	Mean	Standard deviation
Kidney	12	134-277	190	49
Liver	12	118-297	201	52
Muscle	12	222-298	250	23
Heart	12	111-136	126	7

TABLE 5c

TISSUE ZINC IN THE ELDERLY (WEST OF SCOTLAND)
by flame atomic absorption spectroscopy (Martin et al., 1991) (mg/kg dry wt.)

Tissue	Number	Range	Mean	Standard deviation
Kidney	33	94-44	190	84
Liver	33	142-593	305	108
Muscle	33	157-567	255	78
Heart	33	78-164	124	19

The duplicate biopsy was used for histology, determination of alcohol dehydrogenase activity and of total protein content.

Results were expressed as μg Zn/g dry weight, wet weight, mg of protein and as a zinc magnesium ratio. The liver zinc content was significantly reduced in the alcoholic cirrhotic patients whatever convention was used. For example, the controls had a mean value of 266 μg Zn/g dry weight whereas the patients with liver disease had a mean of 96 μg Zn/g dry weight and this was statistically significant.

ZINC IN HAIR

There is an advantage in using a readily obtained sample of head and/or body hair for trace metal analysis. This is a recognised technique when investigating suspected toxic metal exposure, and has been advocated as an index of nutritional status for the essential trace elements (Valcovic, 1977).

The hair sample is digested as described above for tissue or food samples, and the zinc content determined by flame AAS.

A sample of 50-100 mg of head hair is collected from the nape of the neck if possible at a standardised distance from the scalp. An important decision is whether or not to wash the hair sample prior to analysis. This is considered important when external contamination is suspected. Chittleborough (1980) concludes that a 'no washing policy' is best and avoids the risk of accidental contamination.

The clinical usefulness of the measurement is open to question. McBean et al. (1971) did not find a correlation between plasma/serum Zn concentrations and the amount of Zn in hair samples taken from Iranian children. Hair growth is also affected by the restriction of growth caused by nutritional deficiency and the concentration of zinc may be normal in the hair of children known to be malnourished. Additionally, Chittleborough and Steel (1980) found that facial hair zinc did not vary as dosage with Zn salts was increased.

A further complication is noted by De Antonio et al. (1982) in that they found no correlation between head hair metal content and that of pubic hair. They concluded that variable exogenous contamination by trace metals of hair samples is a major problem.

Nevertheless, the finding of a positive correlation between hair zinc concentration and age, height and weight does suggest that carefully planned studies of zinc accumulation in human hair would be a valuable research adjunct to other indices of zinc nutrition (Anon, 1982a).

ZINC IN PERIPHERAL BLOOD

Peripheral blood is the most widely sampled body tissue because of the ease of access by venesection. The concentration of zinc in whole blood is about 6-7 mg Zn/L. The concentration in the erythrocyte is about 10 mg Zn/L. The white cells individually contain some 25 times as much zinc as a red cell, but the concentration varies with the different types isolated from whole blood. Milne et al. (1965) have evaluated different

procedures and note the wide range quoted in the literature of 5-32 μg Zn/10^6 cells for healthy controls. At present it is difficult to compare results from different groups, although the measurement is of considerable research interest. In a survey of methods for the determination of zinc in different components of peripheral blood Ruz et al. (1992) found that while neutrophil zinc content did not reflect a diet low in available zinc that the alkaline phosphatase activity of red cell membranes did offer potential as an index of zinc status.

The concentration of zinc in plasma/serum is lower than in the cellular components of blood, and for healthy persons is around 1 mg/L.

This is by far the most commonly used test of zinc status.

PLASMA/SERUM ZINC - PRACTICAL CONSIDERATIONS

Venesection, using a stainless steel needle and plastic syringe should be performed with minimal venous stasis, since this will alter the concentration of protein bound zinc (Walker et al., 1979). At least 5 mL should be obtained from an adult and 2 mL from a child. The whole blood is ejected gently from the syringe into a plain plastic or glass tube and allowed to clot. The serum is separated by centrifugation and the sample stored at 4°C - 10°C. Care is taken to remove any red cells and serum showing any visible haemolysis must be rejected. Alternatively, the blood can be collected in a tube with anti-coagulant (lithium heparin is suitable) and the plasma removed after centrifugation. The yield of plasma is greater than that for serum and the chances of haemolysis reduced. The zinc concentration in serum is said to be 15% higher than that in plasma due to the release of zinc from platelets during clotting. We do not find an important difference. However, for sequential studies it may be advisable to standardise on one or the other. The blood sample should be obtained from the patient in the morning after an overnight fast. There is a pronounced diurnal variation and a fall in plasma zinc concentration after meals. These physiological changes are not always recognised and are important when considering differences between patient groups and controls.

Sample Preparation for Flame AAS

Plasma/serum samples require to be diluted before determination by flame AAS. The uptake rate of the nebuliser system is lower when dilute protein solutions are aspirated compared to aqueous calibration standards. This effect differs between instruments of different manufacture, being dependent upon nebuliser tube diameter and the gas flow and pressure. The effect can be overcome in a number of ways. At dilutions of plasma/serum of greater than 1 in 20, the viscosity of the diluted solution approaches that of water. This reduces the concentration of zinc in the test solution to around 0.05 mg/L and requires considerable scale expansion and a specially modified instrument (Dawson and Walker, 1969).

A suitably viscous but inert fluid such as glycerol can be added to the calibration standards at a concentration which equalises the uptake rates. This is the basis of a Proposed Selected Method (Smith et al., 1979). This has been criticised by Kelson (1980)

who claims better recoveries of added zinc when a deproteinisation with trichloroacetic acid (TCA) is used to remove the protein from plasma prior to zinc analysis.

There are problems with TCA in that this reagent contributes variably to the reagent blank and appears to depress the zinc absorbance in the air acetylene flame (James and MacMahon, 1971).

Another approach is to dilute the plasma/serum sample in an aqueous solution of an organic alcohol such as n-butanol or n-propanol. This reduces the viscosity of the test solution and enhances the zinc absorbance slightly. A dilution of 1 in 5 or so is suitable and this ensures reasonable signal to noise ratio (Peaston, 1973).

Even although the chemical interferences on the zinc absorbance in an air-acetylene flame are few, some workers advocate the addition of Na and K to the aqueous standards to compensate for 'ionisation' effects. In a detailed comparison of the performance of the commonly used AAS methods in an inter-laboratory quality control programme, Taylor and Bryant (1981) found that none of the methods was markedly superior to the others in terms of precision. They noted that the TCA method gave a higher mean result for plasma/serum zinc and that there was no evidence to support the addition of Na and K to the calibration solutions.

Analytical Performance of a Routine Method

The procedure we use is to dilute 0.5 mL of plasma/serum 1 in 6 with n-propanol (10%) using an auto-diluter (Hamilton 400). A smaller volume of plasma/serum can be used where small samples of blood are obtained as in paediatric cases. Then 100 μL of plasma/serum is diluted manually with the organic alcohol solution. The AAS instrument (e.g. PE 3030) is set to the optimum conditions for zinc absorption in the air-acetylene flame at a wavelength of 213.9 nm. Calibration is with dilute zinc nitrate from a stock solution of zinc nitrate containing 1 g Zn/L. A linear plot of zinc absorbance against zinc concentration is obtained and the concentration of the unknown test solutions calculated.

At a plasma/serum concentration of 0.98 mg/L, the within batch precision was found to be 2.1% ($n = 48$). Internal quality assurance is assessed by use of samples taken from a pooled collection of bovine serum, and the between batch precision is calculated monthly. Typical findings at a mean concentration of 1.14 mg Zn/L are an RSD of 1.8%. External Quality Assurance is by participation in a UK inter-laboratory scheme (Robens Institute, University of Surrey).

Recent AAS Developments

Advantage can be taken of the 1000 times gain in sensitivity for zinc determinations offered by graphite furnace AAS methods (GF-AAS). This technique has been used by Shaw et al. (1982) and by Viera and Hansen (1981) to determine zinc concentrations in small samples of plasma/serum (10-20 μL). Dilution of 100 fold can be used but the procedure requires the preparation of complex standards due to serious chemical interferences. Another potential application of GF-AAS is in the study of the distribution of zinc

in various fractions of plasma/serum.   Following gel filtration and/or affinity chromatography the zinc concentration in the greatly diluted plasma/serum sample can best be determined by GF-AAS (Gardiner et al., 1981; Foote and Delves, 1984). Once again because of the variable suppressive effects upon zinc absorbance, it is necessary to use matrix matched standards.

Although GF-AAS is useful it should only be attempted for zinc analysis when necessary because of the serious calibration problems and the difficulty in obtaining reagents and solvents of sufficient purity to work at the high dilution required by the short linear working range of the method. Recently, it has been shown by Brown and Taylor (1984) that conventional flame AAS can be improved in sensitivity by fitting a slotted quartz tube on the burner head. This allows the determination of zinc on small samples (20-50 μL) using aqueous calibration solutions.

Reference Values for Plasma/Serum Zinc Concentrations

The consensus range for plasma/serum zinc concentrations in healthy adults is 0.8-1.2 mg/L (Versieck and Cornelis, 1980). Results above this range are probably due to unrecognised zinc contamination. Other factors to be considered when deriving a reference range are:

1. The technique of venesection. Prolonged venous stasis increases apparent zinc concentration in plasma/serum by concentrating protein by ultrafiltration. Killerick et al. (1980) estimate an increase of 13% after 3 minute stasis. A suitable contamination free method must be used.
2. The timing of the venesection. Samples must be taken at a standardised time, preferably in the morning, after an overnight fast. Samples taken after food show a drop of 19% and the minimum plasma zinc level is at 1900 h (Killerich et al., 1980).
3. The relation between plasma zinc and plasma albumin. Since about 80% of plasma zinc is bound to albumin, allowance should be made for alterations in plasma albumin concentration when considering plasma zinc levels. Killerick et al. (1980) found a 'weak relationship' within the normal range. When we examined 100 samples submitted for routine biochemistry, we found that the plasma albumin zinc relationship could be expressed as [plasma Zn mg/L] = 0.015 [plasma albumin g/L] + 0.224. The correlation was statistically significant (r = 0.69, $p < 0.001$). Comparisons between control groups and test groups of patients need to take this into account. Venous stasis affects plasma protein concentration as does the posture of the patient when sampling blood. Samples taken from subjects in bed will have a lower protein and hence zinc concentration than those taken from ambulant patients or healthy controls.
4. The age and sex distribution of the controls should match that of the test group. Values for men are a little higher than those for women and zinc concentration tends to fall over the age of 50. This latter effect especially in more elderly populations is also related to lower plasma protein concentrations. Zinc concentrations are lower in plasma of young children reaching adult levels around 3 months of age.

Because of the numerous factors which can affect the reference range, it would be best for each laboratory to determine their own, especially when seeking to demonstrate marginal deficiency.

For 50 adult healthy volunteers (26 men and 24 women) we found a mean ± SD of 0.94±0.12 mg Zn/L. The range was 0.81-1.14 mg Zn/L. Blood was taken with appropriate precautions, at 10.00 am, after an overnight fast.

INTERPRETATION OF PLASMA ZINC CONCENTRATIONS

Acute zinc deficiency, with the signs of skin disease, abdominal pain and the other effects listed in Table 2, is seen at plasma zinc concentrations of less than 0.5 mg/L. The adults who developed the acrodermatitis-like condition during prolonged IVN had plasma zinc levels of 0.3-0.5 mg/L at the time when they began to gain body weight.

Plasma zinc concentrations need to be interpreted in relation to the overall protein or nitrogen balance. When this is positive and the patient is gaining weight but zinc supply is inadequate, plasma zinc concentrations will fall as zinc is taken up for tissue synthesis, and eventually clinical signs will appear. In the catabolic patient losing body weight after trauma, especially with sepsis, zinc is released from tissue, enters the ECF and is excreted in urine (Fell et al., 1973). Plasma zinc levels may be normal or even increased at a time when the patient is in strong negative zinc balance. During the therapeutic starvation of obese patients, when they have developed ketosis, the urinary zinc output rises and plasma zinc concentration can reach 2 mg Zn/L (Al-Shamma et al., 1979). It is in the re-feeding or recovery phase that these deficits in zinc will be revealed and plasma zinc concentrations will fall if adequate supplies are not given.

When plasma zinc values of 0.6-0.8 mg/L are found the interpretation needs to be considered in the light of the following points:
1. Is the patient suffering an infection or inflammatory disease?
2. Was the sample taken within 24-48 h of accidental or surgical trauma?
3. Has allowance been made for changes in plasma albumin concentration? Consider a patient with a measured plasma zinc of 0.6 mg Zn/L and an albumin of 25 g/L, the appropriate plasma zinc, using the equation given earlier would be 0.6 mg Zn/L.

This is likely to be a cause where the primary change is in the plasma albumin concentration and is not due to zinc deficiency per se.

If the plasma albumin was found to be 50 g/L, at an observed plasma zinc of 0.6 mg/L, then the appropriate result should be 0.97 mg/L and in this case zinc deficiency is a possibility, and zinc therapy might be indicated.

When results are close to or within the reference range of 0.8-1.2 mg/L small differences between the test group and the controls are unlikely to have definite biological significance. Factors such as sampling technique, diurnal variation, plasma protein changes and others, are difficult to control. Even in healthy volunteers a variation of 15% was found when taking repeated plasma samples from the same healthy individual (Morrison et al., 1979). Therefore, it is not surprising that there is much contradictory

evidence in the literature. The conclusion by David et al. (1984) that the finding of a decreased plasma zinc concentration in children with atopic eczema is a non-specific consequence of the dermatological condition is reasonable, but does **not** mean that zinc supplementation is inappropriate.

PHARMACOLOGY OF ZINC THERAPY

It is clear that the present laboratory indices of zinc status cannot reliably select causes of mild zinc deficiency. Yet it is often claimed that only such cases will respond to oral zinc therapy. Perhaps the more widespread use of the direct determination of intracellular zinc by the analysis of leukocyte zinc will help, but this is in some doubt (Milne et al., 1984).

The clinical and biochemical response to a reasonable period of zinc therapy may offer the best evidence that a pre-existing deficiency had existed. There is some evidence to suggest that zinc therapy may have a pharmacological action as well as nutritional (Fell, 1985). The dosages of zinc reported in the literature are high, often at 150 mg Zn per day, and continued for several weeks or months. This is well in excess of the amounts needed to correct a mild nutritional depletion. Animal studies in which very high amounts of zinc were fed to mice, indicate that there are effects on tissues which are not pathological and may be beneficial (Aughey et al., 1977). Alterations in immuno-regulation by high doses of zinc may also have a pharmacological basis (Anon, 1982b).

Carefully conducted, double blind placebo controlled, clinical trials are needed to confirm the effect of zinc therapy regardless of the mechanism of action.

The formulation of zinc used should have good patient acceptance to improve compliance. An easily soluble salt such as a zinc citrate/sulphate mix (Solvazinc™) or a zinc gluconate preparation are preferred to capsules of solid zinc sulphate. The zinc should be taken with food to minimise any gastrointestinal irritation. Dosage, in divided amounts, should be 100-150 mg/day and continued for at least three months or longer.

The clinical indices used to monitor progress will vary with the patient group under investigation.

Restoration of the senses of taste and smell are important indices if these can be measured objectively (Henkin and Aamodt, 1983). Improvements in dark adaptation can also be assessed during zinc therapy (Anon, 1982c). In children the rate of growth can be measured and related to the effect of zinc supplementation of any nutritional regimen (Golden and Golden, 1981b). In adults the range of weight gain, especially of lean body mass, could be followed. Functional tests of muscle strength and endurance have been shown to improve in athletes given pharmacological doses of zinc. (Krotkieweski et al., 1982). One of the earliest claims for benefit from zinc therapy was on the acceleration of wound healing in surgical patients. This complex area has been reviewed, and Zielsdorf and Witt (1978) consider that further trials would be worthwhile.

Sequential biochemical measurements could complement such clinical trials. Demonstration of an increasing concentration of plasma zinc is evidence of effective zinc absorption and patient compliance. A rise in the activity of plasma alkaline phosphatase is

also evidence of an effect, provided that the changes caused by liver or bone disease are allowed for. Similarly, sequential measurement of the plasma proteins, albumin and retinol binding protein, can be used as an indication of zinc therapy upon overall protein synthesis.

It must be stressed that self-medication or uncontrolled trial of zinc therapy are not advocated. The side effects of excess zinc dosage are not acute, at least at the levels proposed. Adverse metabolic effects include the induction of a secondary copper deficiency (Fischer et al., 1984). An impairment of laboratory indices of the immune response has been noted at a dosage of 300 mg Zn per day, over a 6-week period (Chandra, 1984).

Nevertheless, as MacFarlane Burnet has observed, "I find it impressive that virtually all the clinical types of zinc deficiency including the genetic ones, have been recognised by the positive therapeutic effect of administering zinc salts by mouth" (Dreosti and Smith, 1983).

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SUBJECT INDEX